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(71) Applicant: **CJ CORP.** [KR/KR]; 500, Namdaemunno 5-ga, Jung-gu, Seoul 100-802 (KR).

(72) Inventors: **LEE, Eun-Jung**; Simgokbon 1-dong, Sosa-gu, Bucheon-si, Gyeonggi-do 422-241 (KR). **PARK, Hyung-Ki**; #601 Sugwang Villart, 1604-3, Seocho 1-dong, Seocho-gu, Seoul 137-071 (KR). **KIM, Hyun-Seok**; #4-1003 Munjeong Siyeong Apt., Munjeong 1-dong, Songpa-gu, Seoul 138-200 (KR). **PARK, Ji-Sook**; #101-801 Siyeong Apt., Junggye-dong, Nowon-gu, Seoul 139-220 (KR). **KIM, Yeon-Hyang**; 100 Sangdo-dong, Dongjak-gu, Seoul 156-030 (KR). **LEE, Hyune-Soo**; #208-202, Sangroksoo Apt., Irwon-dong, Gangnam-gu, Seoul 135-230 (KR). **KOH, Hyung-Kon**; #3-114 Eunma Apt., Daechi 2-dong, Gangnam-gu, Seoul 135-282 (KR). **OH, Myung-Suk**; #374, Dukpyeong-ri, Majang-myeon, Icheon-si, Gyeonggi-do 467-810 (KR).

(74) Agent: **SON, Min**; 19th Floor, City Air Tower, 159-9 Samsung-dong, Gangnam-gu, Seoul 135-973 (KR).

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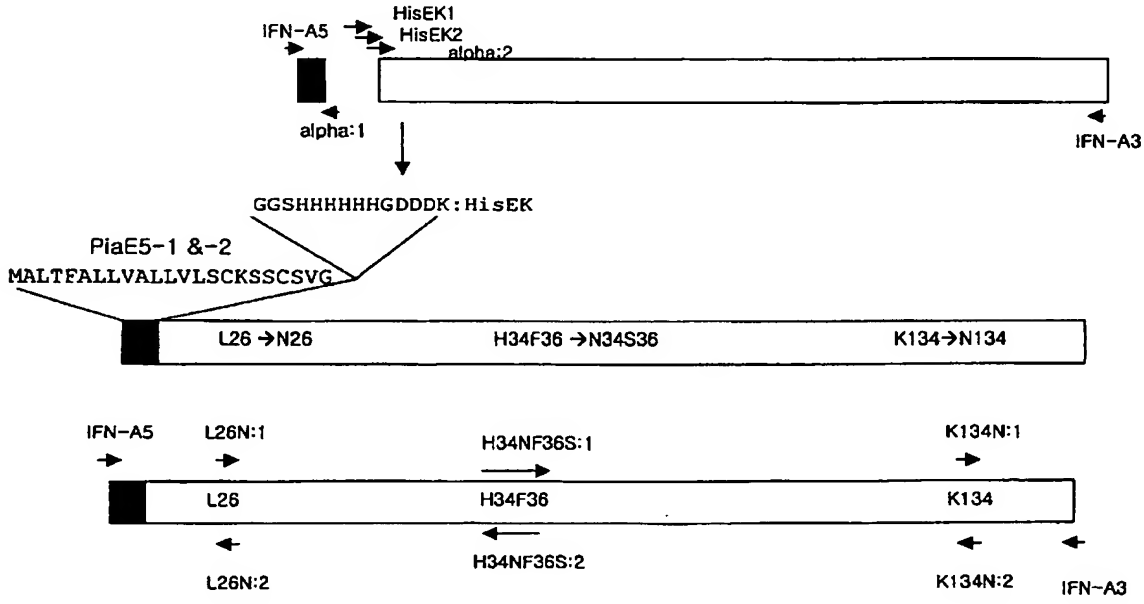

(57) Abstract: The present invention relates to an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at this site and a gene encoding the same, an expression vector comprising the gene, and a method for producing glycosylated human Interferon alpha isoform by transforming or transfecting an eukaryotic cell with the expression vector, culturing the transfected or transformed cell and isolating the glycosylated human Interferon alpha isoform from the culture, the glycosylated human Interferon alpha isoform produced therefrom and a pharmaceutical composition comprising the same.

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GLYCOSYLATED HUMAN INTERFERON ALPHA ISOFORM

TECHNICAL FIELD

The present invention relates to a glycosylated human Interferon alpha isoform. More particularly, the present invention relates to a human Interferon alpha isoform having at least one amino acid modified with another amino acid to increase Asn-X-Ser/Thr(N-X-S/T) sequence at a specific region, thereby increasing in vivo stability, and glycosylated human Interferon alpha isoform thereof.

BACKGROUND ART

Interferon were discovered by Isaacs and Lindenmann (Proc. R Soc. Lond[Biol.], 1957, 147, 258-267) in 1957 and has been known to have strong anti-virus effects.

Interferon is classified into a type I Interferon including Interferon-alpha /-beta and a type II Interferon including Interferon gamma. Interferon-alpha is derived from B lymphocyte or macrophage, Interferon-beta is derived from fibroblast and Interferon-gamma is derived from T lymphocyte.

In human, at least 20 kinds of Interferon-alpha genes and pseudo-genes have been identified. Proteins of these Interferon-alphas are shown to have two disulfide bonds (Cys1-Cys98; Cys29-Cys138) in common. Human Interferon-alpha does not contain a N-type glycosylated bond site but wild type mature protein contains an O-type glycosylated bond at Thr of 106th position (Adolf et al., Biochem. J., 276(Pt 2), 511-518, 1991).

Interferon-alpha can be produced in cells of many tissues, however the

productivity is very low. Generally, it is produced largely in leucocytes such as monocyte/macrophage and B lymphocyte. Here, the proportion of subtypes in produced Interferons varies depending on produced cell types and production conditions. It has been known that the production of Interferons is induced by virus infection. Further, bacteria, mycoplasma, protozoa and the like may induce the production of Interferon and particularly, lipopolysaccharide (LPS) of gram negative bacteria is a strong Interferon inducing agent.

mRNA of Interferon-alpha is continuously produced even in tissues of a normal human (Tovey et al., Proc Natl Acad Sci USA, 1987, vol. 84, 5038-5042). It is believed that this Interferon is an autocrine Interferon playing a important role in growth and differentiation of cell.

The working mechanism in vivo of Interferon is not known yet. According to the report by Branca and Baglioni (Nature, 294, 768-770, 1981), it was shown that Interferon-alpha and –beta bind to the same receptor in human lymphoblastoid cell.

When virus infection takes place in vivo, Interferon is produced and the produced Interferon induced proteins, which perform Interferon's functions. Representative examples of such proteins include 2'- 5'- oligoadenylate synthetase and protein kinase phosphorylation of elF2 (elongation factor2) which is a factor involved in initiation of peptide chain synthesis. The two enzymes are activated double-stranded RNA (Lengyel P., Annu. Rev. Biochem., 51, 251-282, 1982; PestKa et. al., Annu. Rev. Biochem., 56, 727-777, 1982; De Maeyer and De Maeyer-Guignard J., Interferons and other regulatory cytokines, Wiley, New York).

Interferon is clinically applied in treatment of chronic active hepatitis B, acute viral encephalitides, nasopharyngeal carcinoma and the like.

Since most of bioactive proteins used as medicaments shows low stability in living bodies, patients who need the bioactive proteins should frequently receive excessive amounts to maintain a certain level of the proteins so that they can work. Therefore, patients suffers pain and inconvenience and it is desired to produce a bioactive protein having in vivo stability enhanced to alleviate the suffering of these patients.

International Patent Application Publication No. WO 98/48840 discloses a preparation of Interferon alpha conjugated with polyethyleneglycol as a polymer to increase invito stability of bioactive proteins or US PAT No. 6,399,103 discloses a preparation of a medicament by microcapsulation of human growth hormone. However, these methods accompany complicated processes involving primary production of a protein from a microorganism, followed by purification, and subsequent addition reactions. Also, a cross-linking may takes place at a undesired site and homogeneity of the final product may cause a problem. Another approach is a method using glycosylation. Cell surface proteins and secretion proteins produced by eukaryotic cells can be modified by glycosylation. It is known that the glycosylation can affect not only physical properties of a protein but also stability and functions of a protein in living bodies.

DISCLOSURE OF THE INVENTION

Therefore, it is an object of the present invention to readily produce a target protein from a cell line by glycosylation at human Interferon alpha using gene recombinant

technology and prepare a protein which increased in vivo stability.

In one aspect, the present invention provides an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

In another aspect, the present invention provides a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In a further aspect, the present invention provides an expression vector comprising a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In yet further aspect, the present invention provides a transformed or transfected host cell with an expression vector comprising a gene encoding an amino acid-modified

human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In yet further aspect, the present invention provides a method for preparing glycosylated human Interferon alpha comprising culturing a transformed or transfected host cell with an expression vector comprising a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site in a suitable medium under suitable conditions to isolate glycosylated human Interferon alpha isoform.

In yet further aspect, the present invention provides glycosylated human Interferon alpha isoform obtainable by additional glycosylation of an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In yet further aspect, the present invention provides a pharmaceutical composition comprising a glycosylated human Interferon alpha isoform obtainable by additional glycosylation of an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site and a pharmaceutically acceptable carrier.

In yet another aspect, the present invention provides a synthetic oligodeoxynucleotide used as a primer for production of a glycosylation site in human Interferon alpha protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings in which:

Fig. 1 shows the sequence of human Interferon alpha gene and protein. The arrows or straight lines over the sequence represent regions of a helical configuration in the three-dimensional structure of human Interferon alpha protein, the arrow direction represents the direction of the helix according to the order of the amino acid sequence.

Arginine, 23rd amino acid of mature Interferon alpha, has a DNA sequence different from that of the known to the art but encodes the same amino acid. Before the modification, 106th threonine of the mature Interferon alpha protein is a site where glycosylation (O-type) takes place upon production of a human-derived or eukaryotic cell;

Fig. 2 shows a site in the protein structure of human Interferon alpha where amino acid modification of glycosylation according to the present invention takes place, in which the site contains the pre-sequence, 6 histidines as amino acids capable of bonding to a metal ion for readiness of purification and an enterokinase digested site (4 aspartic acids and subsequent lysine sequence);

Fig. 3 is a schematic diagram to show the method for modifying leucine, 26th amino acid with asparagine;

Fig. 4 is a schematic diagram showing the method for modifying histidine, 34th amino acid and phenylalanine, 36th amino acid with asparagine and serine, respectively;

Fig. 5 is a schematic diagram showing the method for modifying lysine, 134th amino acid of wild-type Interferon alpha with asparagine;

Fig. 6 is a schematic diagram showing the method for simultanenously modifying 26th leucine, 34th phenylalanine and 36th phenylalanine of wild-type Interferon alpha with asparagine, asparagine and serine, respectively;

Fig. 7 is a schematic diagram showing the method for simultaneously modifying 26th leucine and 134th lysine with asparagine, respectively;

Fig. 8 shows the result of western blot on human Interferon alpha derivatives.

The primary antibody is a monoclonal antibody against for human Interferon alpha and the secondary antibody is an antibody of rabbit antibody against mouse immunoglobuline bonded to HRP enzyme. Here, 1 represents a marker, 2 represents O-glycosylated IFN-alpha, 3 represents L26N mutant, 4 represents L26N/H34NF36S mutant, 5 represents H34NF36S mutant, 6 represents K134N mutant and 7 represents L26N/K134N mutant; and

Fig. 9 is a graph showing the residual concentration of human Interferon alpha derivatives in mouse according to elapsed time.

BEST MODES FOR CARRYING OUT THE INVENTION

The term "isoform of human Interferon alpha" used herein refers to an analogue or mutant having one or more of inherent amino acid sequence residue of wild-type human Interferon alpha modified with another amino acid while maintaining its inherent activities.

The three letters (single letter) of amino acids used herein mean the following acids according to standard abbreviation regulation in the biochemistry field:

Ala(A): alanine; Asx(B): asparagine or aspartic acid; Cys(C): cystein;

Asp(D): aspartic acid; Glu(E): glutamic acid; Phe(F): phenylalanine;

Gly(G): glycine; His(H): histidine; Ile(I): isoleucine; Lys(K): lysine; Leu(L): leucine; Met(M): methionine; Asn(N): asparagine; Pro(P): proline;

Gln(Q): glutamine; Arg(R): arginine; Ser(S): serine; Thr(T): threonine; Val(V): valine; Trp(W): tryptopan; Tyr(Y): tyrosine; Glx(Z): glutamine or glutamic acid.

"(amino acid single letter)(amino acid position)(amino acid single letter)" used herein refers that the former amino acid at the corresponding amino acid position of human Interferon alpha is substituted with the latter amino acid. For example, L26N indicates that leucine corresponding No. 26 of whild-type human Interferon alpha is substituted with asparagine.

In the present specification, a primer for production of glycosylation site is expressed as "(amino acid single letter)(amino acid position)(amino acid single letter) 1 or 2", in which 1 is a primer complementary to a single strand template proceeding 5'→3' direction in a double strand template and 2 is a primer complementary to a single strand template proceeding 3'→5' direction in a double strand template.

Secretion proteins produced by eukaryotic cells as a host cell may be modified by at least one oligosaccharide. It was known that such modification called glycosylation may enormously affect to physical properties of the proteins and be critical in stability, secretion and location in a cell of the proteins. Proper glycosylation may be necessary for biological activity. In practice, when a gene derived from an eukaryotic cell is expressed in bacterium lacking a intracellular process to glycosylate a protein, a protein with deteriorated activity due to the lack of glycosylation is produced.

The glycosylation takes place at a certain position depending on a polypeptide backbone, typically including two types. One is O-type glycosylation which involves binding of oligosaccharide to –OH group of serine or threonine residue and the other is N-type glycosylation which involves binding of oligosaccharide to –NH group of asparagine residue. Particularly, the N-type glycosylation takes place in case having a specific amino acid sequence and the sequence is known as Asn-X-Ser/Thr(N-X-S/T), in which X may be any amino acid except for proline. The N-linked oligosaccharide and the O-linked oligosaccharide have different structures and residues found in each type are also different from each other. For example, in the O-linked saccharide residue, N-acetylgalactosamine is always bonded to serine or threonine while in the N-linked saccharide residue, N-acetylglucosamine is always bonded to asparagines. The O-linked oligosaccharide generally comprises 4 or less of saccharide residues while the N-linked oligosaccharide always contains N-acetylglucosamine and mannose and comprises at least 5 saccharide residues.

The present invention relates to an amino acid-modified human Interferon alpha isoform to increase in vivo stability of a protein comprising at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

The present inventors have discovered that glycosylation by amino acid modification may be induced at any region except for the helical region in the amino acid sequence of human Interferon alpha protein.

In one embodiment, the present invention is directed to an amino acid-modified

human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

In a preferred embodiment, the present invention is directed to an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52); and

-Lys134-Ser137(Lys134-Tyr-Ser137).

In a more preferred embodiment, the present invention is directed to an amino

acid-modified human Interferon alpha isoform having 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or having all of these modifications.

The present invention comprising modifying at least one nucleotide so that N-type glycosylation may occur on a DNA sequence encoding human Interferon alpha to have an additional glycosylation site, introducing the DNA glycosylation to an eukaryotic cell carrying out the glycosylation, followed by expression so that the additional glycosylation naturally occurs. The additionally glycosylated human interferon alpha according to the present invention is achieved by modifying the DNA sequence so that Asn-X-Ser/Thr(N-X-S/T) sequence is increased.

In one embodiment, the present invention is directed to a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

In a preferred embodiment, the present invention is directed to a gene encoding an amino acid-modified human Interferon alpha isoform having at least one amino acid modified with another amino acid so that the Asn-X-Ser/Thr(N-X-S/T) sequence is increased at the following amino acid residue positions:

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52); and

-Lys134-Ser137(Lys134-Tyr-Ser137).

In a more preferred embodiment, the present invention is directed to directed to a gene encoding an amino acid-modified human Interferon alpha isoform having 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or having all of these modifications.

In one embodiment of the present invention, the gene encoding human Interferon alpha is obtained from human Interferon alpha-producing strain for animal cell expression. For gene cloning and separation, methods known to the art may be used.

The human Interferon alpha gene obtained from the above may be modified in at least one selected codon. In the present specification, modification may be defined as substitution of one or more codon(s) on a gene encoding human Interferon alpha to make a change in the amino acid sequence of human Interferon alpha. More particularly, it refers to substitution of at least one amino acid with another amino acid so that the Asn-X-

Ser/Thr(N-X-S/T) sequence for additional N-type glycosylation is formed on the amino acid sequence of human Interferon alpha. For example, in Example 3 of the present invention, when 26th leucine is substituted with asparagine, since 28th amino acid is serine, the Asn-X-Ser/Thr(N-X-S/T) sequence is formed, whereby an additional N-type glycosylation may take place. Also, when 34th histidine and 36th phenylalanine are substituted with asparagine and serine, respectively, the Asn-X-Ser/Thr(N-X-S/T) sequence is formed, whereby an additional N-type glycosylation may take place. Further, when 134th lysine is substituted with asparagine, since 136th amino acid is serine, the Asn-X-Ser/Thr(N-X-S/T) sequence is formed, whereby an additional N-type glycosylation may take place.

In one embodiment, a synthetic oligonucleotide comprising a codon encoding a desired amino acid modification in human Interferon alpha is constructed. Typically, an oligonucleotide having a length of about 25 nucleotides is used. Though an oligonucleotide with a more shorten length can be employed, the optimal oligonucleotide is to have 12 to 15 nucleotides complementary to a template at both sides of the nucleotides encoding the modification. Such oligonucleotide may be sufficiently hybridized to the template DNA. The synthetic oligonucleotides used for production of an additional glycosylation site in the present invention are shown in Table 2. These oligonucleotide can be synthesized by technologies known to the art.

In one embodiment of the present invention, there is provided a human Interferon alpha isoform DNA with one amino acid modified. PCR is conducted using human Interferon alpha DNA is used as a template and a synthetic oligonucleotide encoding a

modification as a primer. In the heating step of PCR, the double stranded template is separated and to each of the single strand template, a complementary primer is hybridized. DNA polymerase bonds nucleotides complementary to the template from –OH group of the primer encoding the modification in 5′ → 3′ direction. Consequently, the second strand contains the primer encoding the modification and thus encodes the desired modification on a gene. The second strand serves as a template DNA in the repeated replication steps of PCR and the gene encoding the modification will be continuously amplified. For example, in Example 3 of the present invention, in order to modify leucine, the 26th amino acid residue with asparagine, PCR is conducted using wild-type Interferon alpha DNA as a template and primer pairs of IFN-A5' and L26N2, and L26N1 and IFN-A3'. As a result, two DNA segments, in which 26th amino acid position is changed to a codon corresponding to asparagine instead of leucine, are obtained. Then, secondary PCR is conducted using the two DNA segments thus obtained and IFN-A5' and IFN-A3' as a primer pair to obtain a modified gene of IFN-alpha-L26N, in which 26th amino acid is modified with asparagine instead of leucine so that a glycosylation may occur.

In another embodiment of the present invention, there is provided a human Interferon alpha isoform comprising two or more amino acid modifications. A mutant having two or more amino acids modified is constructed by various methods. When the two or more amino acids to be modified are adjacent to each other on a polypeptide, they can be simultaneously modified using an oligonucleotide having all the amino acid modifications encoded. Therefore, the construction of the mutant is the same with the method for construction of a human Interferon alpha gene with one nucleotide modified

except that an oligonucleotide having two or more amino acid modifications as a primer. However, when the two or more amino acids are far from each other on a polypeptide (spaced by 10 or more amino acids), it is impossible to construct an oligonucleotide having all the desired modifications encoded.

Instead, other methods should be introduced. The fist method is to construct individual oligonucleotides containing each amino acid modification. If the oligonucleotides are simultaneously annealed to a single strand template DNA, the second strand DNA synthesized from the template will encode all the desired amino acid modifications. Another method in the present invention includes two or more times mutagenesis to produce such an isoform. In the first mutagenesis, wild-type DNA is used as a template and an oligonucleotide containing the first desired amino acid modification is annealed to the template to form a heterogeneous DNA (heteroduplex). In the second mutagenesis, the modified DNA, prepared in the first mutagenesis, is used as a template. Thus, this template already contain at least one modification. To this template, an oligonucleotide containing an additional amino acid modification is annealed and the resulting DNA have all the modifications of the first and second mutagenesis encoded.

The resulting DNA can be used as a template in the third mutagenesis. In summary, the foregoing method for modifying two or more nucleotides is to repeat a method for modifying one nucleotide several times. For example, in Example 3 of the present invention, to modify leucine, 26th amino acid of wild-type Interferon alpha protein with asparagine and 134th amino acid, lysine with asparagine at the same time, firstly 134th position is modified and a modification of 26th amino acid is conducted using the

previously modified DNA as a template. As a result, a human Interferon alpha gene having the two residues modified is obtained.

The DNA sequences encoding the human Interferon alpha isoforms according to the present invention can be synthesized by any standard method known to the art, for example using an automatic DNA synthesizer (ex. Biosearch, Applied Biosystem™).

The glycosylated isoform according to the present invention is typically produced by (a) inserting the DNA sequence encoding human Interferon alpha isoform to a vector having one or more expression control sequence operatively linked to the DNA sequence to control its expression, (b) transforming or transfecting a host with the resulting recombinant expression vector, (c) culturing the transformed or transfacted cell in a proper medium and condition to express the human Interferon alpha isoform DNA sequence, followed by isolation of the glycosylated human Interferon alpha isoform.

In connection with this, the present invention provides a host cell transformed or transfected with the recombinant expression vector containing the DNA sequence encoding the human Interferon alpha isoform.

Of course, it should be understood that all the vectors and expression control sequences do not equally play their functions to express the DNA sequence according to the present invention. Similarly, all the host cells do not equally play their functions for the same expression system. However, those skilled in the art may properly select a vector, expression control sequence and host cell without departing the scope of the present invention while not bearing excessive experiments. For example, in selection of a vector, a host cell must be considered. This is because the vector should be replicated therein.

Also, the replication number and ability to control the replication number of a vector and expression of other proteins encoded by the vector, for example antibiotic marker should be considered. In selection of an expression control sequence, various factors should be considered. For example, relative strength of the sequence, controllability and compatibility with the DNA sequence of the present invention, particularly with respect to a possible two-dimensional structure should be considered. Also, in selection of a host, compatibility with a selected vector, toxicity, secretion properties and ability to correctly fold a polypeptide of the product encoded by the nucleotide sequence, fermentation or cultivation requirements and conditions and readiness of purification of the product encoded by the nucleotide sequence.

The term "vector" used herein refers to a DNA molecule as a carrier capable of stably carrying a foreign gene into a host cell. In order to be a useful vector, a vector can be replicated, has a means to be introduced into a host cell and to detect its own presence.

The term "recombinant expression vector" refers to a cyclic DNA molecule, in which a foreign gene is operably linked to a vector so that the gene can be expressed in a host cell. The recombinant expression vector can be produced as several copies and heterogeneous DNA inserted therein. As well-known to the art, in order to increase expression level of a transfected gene in a host cell, the gene should be operably linked to open frame expression control sequence which can work in a selected expression host. Preferably, the gene is contained in an expression vector comprising a selection marker and replication origin. When an expression host is a eukaryotic cell, the expression vector should further comprise an expression marker useful in the eukaryotic expression host cell.

Various expression vectors can be used to express the DNA sequence encoding the human Interferon alpha isoform. Preferably, an expression vector suitable for an eukaryotic host cell since glycosylation takes place on the human Interferon alpha isoform.

Examples of expression vectors useful for eukaryotic host cells include expression control sequences derived from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific examples of the vectors include pCDNA3.1(+)\Hyg(Invitrogen, Carlsbad, Calif., USA) and pCI-neo(Stratagen, La Jolla, Calif., USA). Expression vectors useful for yeast cells include 2μ plasmid and derivatives thereof, POT1 vector(U.S. Pat. No. 4,931,373) and pPICZ A, B, or C(Invitrogen). Expression vectors useful for insect cells include pVL·941, pBluebac 4.5 and pMelbac(Invitrogen).

"Expression control sequence" refers to nucleic acid sequences necessary or beneficial to polypeptide expression. Respective expression control sequences can be a native or foreign on a nucleic acid encoding a polypeptide. Examples of the control sequence include, but are not limited thereto, leader sequence, polyadenylated sequence, propeptide sequence, promoter, enhancer or upstream activation sequence, signal peptide sequence and transcription termination factor. An expression control sequence contains a promoter.

In order to express the DNA sequence of the present invention, various expression control sequences can be used as a vector. Examples of expression control sequence suitable to instruct expression in mammal cells include early and late promoters of SV40 and adenovirus, MT-1(metallothioneine gene) promoter, human cytomegalovirus early

gene(CMV), Raus sarcoma virus (RSV) promoter and human Ubiquitine C (UbC) promoter. In order to further improve expression in mammal cells, synthetic intron can be inserted into a non-transcription region of a nucleotide sequence encoding a polypeptide.

Examples of expression control sequences suitable to instruct expression in insect cells include polyhedrin promoter, P10 promoter, baculovirus 39K delayed-early gene promoter and SV40 polyadenylation sequence. Examples of expression control sequences usable in yeast cells include a promoter of α-mating system, yeast triose phosphate isomerization enzyme (TPI) promoter and ADH2-4c promoter. Examples of expression control sequences suitable to instruct expression in fungus cells include ADH3 promoter and termination factor.

Other usable component of a vector used in practicing the present invention is a signal peptide. This sequence is typically located at 5' of a gene encoding a protein and is thus, transcripted to amino terminus of the protein. Presence or absence of a signal peptide varies depending on an expression host cell used in production of a polypeptide to be expressed (according to whether the polypeptide to be expressed is intracell or extracell polypeptide) and preference of recovering secreted products. The signal peptide exists when a polypeptide is secreted from a expressed cell. If the signal peptide exists, it should be recognized by a cell selected for expression of a polypeptide. The signal peptide peptide can be homologus to a polypeptide (typically associated with the polypeptide) or heterologus to a polypeptide (derived from one other than the polypeptide) and can be homologus or heterologus to a host cell.

A nucleic acid is "operably linked" to another nucleic acid when they are arranged

in a functional relationship. This means that an appropriate molecule (for example, a transcription activator) binds to a regulatory sequence(s), a gene or a regulatory sequence(s) linked in such a way that the expression of the gene is modulated. For example, when a pre-sequence or secretory leader participates in secretion of a mature protein, they are operably linked to the promoter. When a promoter affects transcription of a coding sequence, the promoter is operably linked to the coding sequence. When a ribosomal binding site is located at a place capable of reading a coding sequence, the ribosomal binding site is operably linked to the coding sequence. Generally "operably linked" means that to contact with a linked DNA and a secretory leader and to be in a reading frame. However, the enhancer does not need to contact. The linkage of these sequences are effected by ligation (linkage) in a convenient restriction enzyme site. If such a site does not exist, a conventionally synthesized oligonucleotide adaptor or linker may be used.

Construction of a suitable vector comprising a gene encoding the human Interferon alpha isoform and the foregoing components (i.e. a control sequence) can be performed using a basic recombinant technology. In order to prepare a desired vector, respective DNA segments are firstly digested with restriction enzymes and then ligated to each other considering a particular order and orientation.

DNA can be digested using a particular restriction enzyme in a proper buffer.

Typically, about 0.2~1ug of a plasmid or a DNA segment is used along with about 1 to 2 units of a needed restriction enzyme in about 20 μl of buffer. A proper buffer, DNA level, incubation time and temperature are specified by a manufacturer of the restriction enzyme. Typically, it is suitable to incubate for about 1 to 2 hours at 37℃,

though some enzymes need a higher temperature. After incubation, enzymes and other impurities can be removed by extraction the digestion solution with a mixture of phenol and chloroform and DNA can be recovered from the aqueous layer by precipitation with ethanol. Here, ends of the DNA segments are compatible with each other so that the DNA segments can form a functional vector.

The digested DNA segments are classified and selected according to their sizes by electrophoresis. DNA can be electrophoresed through agarose or polyacrylamide matrix. Selection of the matrix can be determined by a size of the DNA segment to be isolated. After electrophoresis, DNA is extracted from the matrix by electroelution. When a low-melting agarose is used, agarose is melted and DNA is extrated therefrom.

The DNA segments to be ligated should be added to the solution in an equal molar amount. The solution contains ATP, ligase buffer, ligases such as about 10 units of T4 ligase per DNA 0.5ug. In order ligate a DNA segment to a vector, the vector should be linearlized through digestion with a suitable restriction enzyme. The linearlized vector is treated with alkaline phosphatase or calf intestinal alkaline phosphatase. The treatment with phosphorylase inhibits self-ligation of a vector during the ligation step. The recombinat expression vector prepared by the above-described method is then used to transform or transfect a host cell.

In selection of a host cell, a host cell having a high DNA introduction efficiency and showing a high expression efficiency of the introduced DNA. Particualrly, in the present invention, eukaryotic host cells to perform glycosylation to the human Interferon alpha isoform is used. Suitable examples of yeast host cells include Saccharomyces and

Hansenula strains. Suitable examples of fungus host cells include Tricoderma, Fusarium and Aspergillus strains. Suitable examples of insect host cell include Lepidoptora cell lines such as Sf9 or Sf21. Suitable examples of mammal host cells include CHO cell line, COS cell lines such as COS 1, COS 7, BHK cell lines and animal cells such as mouse cells, tissue cultured plant cells and human cells.

Polynucleotide can be introduced to a host cell by methods described in basic experiment manuals such as [Davis et al., Basic Methods in Molecular Biology(1986)] and [Sambrook et al., (1989) Molecular Cloning 2nd Edition]. Preferred methods for introducing a polynucleotide to a host cell include, for example, calcium phosphate transfection, DAEA-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

In the production method of the present invention, host cells are cultured in a nutrient medium suitable for polypeptide production using a known technoloty. For example, cells can be cultured in a suitable medium in a fermenter for laboratory or industry under conditions acceptable for expression and/or secretion of a polypeptide by small-scale or large scale fermentation, shake flask culture. The cultivation is conducted in a proper nutrient medium comprising carbon, nitrogen supply source and inorganic salts using a known technology. The medium is well known to those skilled in the art and is commercially available or can be produced. When a peptide is directly secreted to a nutrient medium, the polypeptide can be directly isolated from the medium. When a polypeptide is not secreted, it can be isolated from cell lysate

A polypeptide can be isolated by a method known to the art. For example, it can be isolated from a nutrient medium by traditional methods including, but not limited thereto, centrifugation, filtration, extraction, spray drying, evaporation or precipitation. Further, a polypeptide can be purified by various methods known to the public including chromatograph (ex. Ion exchange, affinity, hydrophilic, hydrophobic, size-exclusion), electrophoresis, fractional solubility (ex. Ammonium sulfate precipitation), SDS-PAGE or extraction.

The present invention provides a glycosylated human Interferon alpha isoform with an additional glycosylation obtainable through the above described procedure. In the present specification, the glycosylated human Interferon alpha isoform can be defined as an expression product obtained by introducing a human Interferon alpha gene which is modified to increase the Asn-X-Ser/Thr(N-X-S/T) sequence into an eukaryotic host cell, follwed by expression so that glycosylation can spontaneously occur. That is, it refers to a heterogenous molecular formed by covalent bonding of sugar residues to asparagine –NH group of Asn-X-Ser/Thr(N-X-S/T), an additional glycosylation site of the human Interferon alpha isoform.

The present invention provides a pharmaceutical composition comprising glycosylated human Interferon alpha isoform with an additional glycosylation and a pharmaceutically acceptable carrier. A therapeutic preparation of the glycosylated human Interferon alpha isoform for therapeutic administration can be formulated into lyophilized cake and aqueous solution combining any pharmaceutically acceptable carrier, excipient, stabilizer and the glycosylated human Interferon alpha isoform having a desired purity. A

preparation for parenteral administration can be prepared by combining the glycosylated human Interferon alpha isoform with a pharmaceutical carrier into a formulation, which can be administered (solution, suspension or emulsion).

The pharmaceutically acceptable carrier, excipient or stabilizer do not shown toxicity to a patient who receiving them at a dose and concentration to be administered and are compatible with other ingredients. For example, the preparation should not contain an oxidant or other substances, which are known as being harmful to a polypeptide.

Suitable carriers include buffers such as phosphoric acid, citric acid and other organic acids; antioxidants such as ascorbic acid; low-molecular polypeptides; proteins such as serum albumin, gelatin and immunoglobulin; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, arginine or lysine; monosaccharides such as mannose or dextrin, disaccharides, other carbohydrates; chelating factors such as EDTA; metal ions such as zinc, cobalt or copper; sugar alcohols such as manitol or sorbitol; salt-forming counter ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

In order to use the glycosylated human Interferon alpha isoform for therapeutic administration, it should be sterilized. The sterilization can be readily accomplished by filtration through a sterile filtration membrane.

The therapeutic composition of the glycosylated human Interferon alpha isoform is typically stored in a container having a sterile access port, such as, for example, vascular injection bag having a cap through which a subcutaneous injection needle can pass or vial. The human Interferon alpha will be stored as an aqueous solution or lyophilized preparation

in a single dose or multi-dose container, for example, a sealed vial or an ampoule. In case of the lyophilized preparation, 5 ml of sterilized and filtered 1%(w/v) human Interferon alpha aqueous solution is filled in a 10 ml-vial and the mixture is lyophilized. The injection can be prepared by reconstruction of the lyophilized human Interferon alpha with bacteriostatic Water-for-Injection.

The glycosylated human Interferon alpha isoform can be directly administered to animals by a proper technology including paranteral administration, or locally or systemically administered. A particular administration route can be determined depending on, for example, a patient's case history including side effects which are recognized or expected by the human Interferon alpha. Examples of the parenteral administration include subcutaneous, intramuscular, intravascular, intraarterial, intraperitoneal administration. Most preferably, the administration is carried out by sustained injection (ex. a mini pump such as osmosis pump) or injection through, for example, intravascular or subcutaneous route. The glycosylated human Interferon alpha isoform is preferably administered subcutaneously.

The glycosylated human Interferon alpha isoform is administered to a patient in a therapeutically effective amount. The term " therapeutically effective amount" can be defined as an amount sufficient to show a desired therapeutic effect in a given condition and administration method. The human Interferon alpha composition for treatment should be prepared and administered considering particular conditions to be treated, clinical conditions of individual patients (particularly side effects upon treatment with human Interferon alpha along), delivery location of the glycosylated human Interferon alpha

isoform, administration method, administration schedule, other factors known to those skilled in the art and being consistent with preferred medical practices. The therapeutically effective amount in the treatment with the glycosylated human Interferon alpha isoform is determined by the foregoing matters. A daily effective amount of the glycosylated human Interferon alpha isoform according to the present invention is in the range of about 2×10^6 units to 500×10^6 units.

Now, the present invention will be described in further detail by the following examples. However, the examples are only for illustration of the present invention and the present invention is not limited thereto.

<Example 1>

Preparation of human Interferon alpha gene

As human Interferon alpha gene, modified Interferon alpha-producing strain possessed by the applicant was used. The Interferon alpha gene possessed by the applicant did not comprise the whole sequence for expression in E. coli. Therefore, PCR using a chemically synthesized oligodeoxynucleotide was conducted to prepare the whole sequence. The human Interferon alpha gene without the whole sequence was amplified using PiaE5-1 and IFN-A5' synthetic oligodeoxynucleotide. The amplified DNA segment was amplified by PCR using a synthetic oligodeoxynucleotide of PiaE5-2 and IFN-A3' to introduce the full-length signal sequence at 5'-end of human Interferon alpha gene. The used synthetic oligodeoxynucleotide are shown in Table 1.

Table 1

Synthetic oligodeoxynucleotide used as primer for construction of whole sequence

Primer Name	primer sequence	SEQ ID NO
PiaE5-1	5-'GTGCTCAGCTGCAAGTCAAGCTGCTCTGTGGGCTGTGATCTGCCTCAAACCCAC-3'	1
PiaE5-2	5'-ATGGCCTTGACCTTTGCTTTACTGGTGGCCCTCCTGGTGCTCAGCTGCAAGTCA-3'	2
IFN-A5'	5'-TCCCAAGCTTATGGCCTTGACCTTTGCTTTACTG-3'	3
IFN-A3'	5'-TGGGATCCTCATTCCTTACTTCTTAAACTTTCTTG-3'	4
HisEK:1	5'-AAGCTTCCCATGGGGGGTTCTCATCATCATCATCATCATGGG -3'	5
HisEK:2	5'-CATCATCATCATCATCATGGGGACGATGACGATAAG-3'	6
Alpha: 1	5'-ACCCCCCATGGAGCCCACAGAGCAGCTTGA-3'	7
Alpha: 2	5'-GGGGACGATGACGATAAGTGTGATCTGCCTCAAACC-3'	8

<Example 2>

Selection of modification site on human Interferon alpha gene

In order to select a site for additional glycosylation on human Interferon alpha, the result of the reference [Walter(Structure(1996) vol.4, 1453)] was used. In selection of a site, firstly, the helical region in the amino acid sequence of human Interferon alpha protein was excluded (Fig. 1). From the sequence with the helical region excluded, a second site was selected, considering that No. 106 threonine residue of wild-type Interferon has O-type glycosylation in three-dimension. From the secondly selected site, a site where N-type glycosylation could be readily converted to a motive was finally selected.

As shown in Fig. 1, the sites to attempt modification for addition of an additional

glycosylation site was L26, H34 and F36, and K134, in which 26th leucine was modified with asparagine, 34th histidine and 36th phenylalanine were modified with asparagine and serine, and the 134th lysine was modified with asparagine. There are shown synthetic oligodeoxynucleotides used for this experiment. The direction of the arrow represents of 5'-> 3' direction of respective oligodeoxy nucleotides.

In order to purify human Interferon alpha protein, an additional amino acid sequence (HisEK) was inserted between the pre-sequence and the amino acid sequence of mature human Interferon alpha protein. The amino acid sequence was M-G-G-S-H-H-H-H-H-H-G-D-D-D-D-K-. By inserting this amino acid sequence, expressed human Interferon alpha derivative protein can isolated by metal affinity column chromatography. The isolated protein was treated with enterokinase and subjected to metal affinity column chromatography to obtain only human Interferon alpha derivative protein.

The insertion of HisEK sequence was conducted by amplifying DNA at the pre-sequence region by PCR with IFN-A5 and alpha:1 primer, followed by digestion with restriction enzyme NcoI. Then, the mature human Interferon alpha gene region was primarily amplified with alpha:2 and IFN-A3. The resulting DNA segment was secondarily amplified with HisEK:2 and IFN-A3 and then with HisEK:1 and IFN-A3 to obtain a DNA segment. The resulting DNA segment was digested with restriction enzyme NcoI and the resulting two DNA segments were joined using T4 DNA ligase.

The joined human Interferon alpha gene was again amplified by PCR using IFN-A5 and IFN-A3 primers. The amplified DNA segment was digested with restriction enzyme HindIII and BamHI and inserted to pcDNA3.1Hygro+ plasmid vector, which had

been digested with the same restriction enzymes, using T4 DNA ligase to form an expression vector.

<Example 3>

Construction of human Interferon alpha isoform

A gene encoding human Interferon alpha having at least one amino acid modified to provide an additional glycosylation site can be modified by PCR using a synthetic oligodeoxynucleotide as a primer. The used synthetic oligodeoxynucleotide are shown in Table 2.

Table 2

Synthetic oligodeoxynucleotide used for production of additional glycosylation

primer name	primer sequence	SEQ ID NO
L26N1	5'-GCACAGATGAGGCGCATCTCTAACTTCTCCTGCTTGAAGGACAGA-3'	9
L26N2	5'-TCTGTCCTTCAAGCAGGAGTTAAGAGAGATGCGCCTCATCTGTGC-3'	10
H34NF36S:1	5'-TTGAAGGACAGAAACGACAGCGGATTTCCCCAG-3'	11
H34NF36S:2	5'-CTTCATCAGGGGAGTCTCGTTCACCCCCACCCC-3'	12
K134N1	5'-ACTCTCTATCTGAAAGAGAAGAACTACAGCCCTTGTGCCTGGGAG-3'	13
K134N2	5'-CTCCCAGGCACAAGGGCTGTAGTTCTTCTCTTTCAGATAGAGAGT-3'	14
IFN-A5'	5'-TCCCAAGCTTATGGCCTTGACCTTTGCTTTACTG-3'	15
IFN-A3'	5-'TGGGATCCTCATTCCTTACTTCTTAAACTTTCTTG-3'	16

(1) Construction of L26N modified human Interferon alpha isoform (Fig. 3)

The human Interferon alpha gene obtained from Example 1 were amplified by PCR with synthetic oligodeoxynucleotide primers, IFN-A5' and L26N2, L26N1 and IFN-A3' to prepare DNA segments. Each of the prepared DNA segments was purified, denatured with 0.2M NaOH/2mM EDTA and subjected to PCR to prepare a gene with an amino acid at a desired site changed (Leu→Asn). As a result, two DNA segments substituted with a codon corresponding to asparagine instead of leucine at the No. 26 amino acid position were obtained. The two DNA segments were subjected to secondary PCR using a primer pair of IFN-A5' and IFN-A3' to obtain a modified gene of IFN-alpha-L26N, in which 26th amino acid is modified with asparagines so that an additional glycosylation can take place.

(2) Construction of H34NF36S modified human Interferon alpha derivative (Fig. 4)

Using the same method for the L26N modified modified human Interferon alpha derivative, human Interferon alpha gene was amplified by PCR with synthetic oligodeoxynucleotides IFN-A5 and H34NF36S:2, and H34NF36S:1 and IFN-A3 to prepare DNA segments.

Each of the DNA segments was purified and subjected to the same method as described above to prepare IFN-alpha H34NF36S modified gene, in which histidine at 34th amino acid position was changed to asparagine and phenylalanine at 36th amino acid position is changed to serine.

(3) Construction of K134N modified human Interferon alpha isoform (Fig. 5)

Using the same method for the L26N modified modified human Interferon alpha derivative, human Interferon alpha gene was amplified by PCR with synthetic oligodeoxynucleotides IFN-A5' and K134N2, and K134N1 and IFN-A3' to prepare DNA segments.

As a result, as shown in Fig. 4, two DNA segments substituted with a codon corresponding to asparagine instead of lysine at 134th amino acid position were obtained.

The two DNA segments were subjected to secondary PCR using a primer pair of IFN-A5' and IFN-A3' to obtain a modified gene of IFN-alpha-K134N, in which 134th amino acid is modified with asparagine so that an additional glycosylation can take place.

(4) Construction of human Interferon alpha derivative with both L26N and H34NF36S modified (Fig. 6)

The same method for the L26N modified modified human Interferon alpha derivative was followed using H34NF36S modified human Interferon alpha derivative.

(5) Construction of human Interferon alpha isoform with both L26N and K134N modified (Fig. 7)

The same method for the L26N modified modified human Interferon alpha derivative was followed using K134N modified human Interferon alpha isoform. In other words, 134th position was modified by the same method as shown in Fig. 5 and using the

product as a template, 26th position was modified by the same method as shown in Fig. 3. As a result, a human Interferon alpha gene with two sites modified at the same time was obtained.

<Example 4>

Transfection into CHO cell and expression

In a 60mm cell culture dish, CHO cells (DG44) were raised to 40-80% confluent (1-4 X 10^5cell/60mm dish). 3 $\mu\ell$ of Superfectin reagent (BM) and 97 $\mu\ell$ of cell culture medium (α -MEM with media, serum-free, antibiotic-free) were thoroughly mixed and human Interferon alpha derivative expression vector DNA (0.1 $\mu g/\mu\ell$ or more, about 2 μg) and vector pLTRdhfr26 (ATCC37295, about 0.2 μg) containing dhfr were added thereto. After the reaction was left for 5 to 10 minutes at room temperature and added to the prepared cells. After one day, the medium was exchanged with a memdium containing 200 $\mu g/m\ell$ of hygromycine (α -MEM without media, 10% FBS) and cultured for about 7 to 10 days. In the medium containing hygromycine at a concentration of 200 $\mu g/m\ell$, cell lines with human Interferon alpha derivative introduced were selected. Each of the selected cell lines was cultured and confirmed for expression of human Interferon alpha derivative by using a human Interferon alpha (Hu-IFN-α) Multi-Specific ELISA Kit (PBL, Product No. 41105-1;).

<Example 5>

Purification of human Interferon alpha derivative

The human Interferon alpha derivatives expressed in CHO cells were purified by condensing the culture fluid using Centriprep (Mw Cut 10,000, Milipore) and subjecting to metal affinity method using ProBond Purification System (Invitrogene).

<Example 6>

Pharmacokynetic test in rat

In order to confirm whether candidates can sustained in actual living bodies, Sprague Dawley rats were used. Amimals were injected with human Interferon derivative in a dose of 1 X 10^6 U/Kg body weight. Each group possessed 4 animals. In order to confirm blood concentration, blood was taken every 30 minutes. The blood samples were analyzed on Human Interferon Alpha(Hu-IFN-α) Multi-Specific ELISA Kit (PBL).

INDUSTRIAL APPLICABILITY

The glycosylated human interferon alpha isoform according to the present invention can has increased in vivo stability and thereby, reduce dose in clinical applications and the frequency of administration.

While the present invention has been described with reference to the particular illustrative embodiments, it is not to be restricted by the embodiments but only by the appended claims. It is to be appreciated that those skilled in the art can change or modify the embodiments without departing from the scope and spirit of the present invention.

What Is Claimed Is:

1. An amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

2. The an amino acid-modified human Interferon alpha isoform according to claim 1, which has 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or has all of these modifications.

3. A gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

4. The gene according toclaim 3, wherein the human Interferon alpha has 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or has all of these modifications.

5. A method for producing a glycosylated human Interferon alpha isoform comprising the steps of: culturing a eukaryotic host cell transformed or transfected with an expression vector comprising a gene encoding the Interferon alpha isoform according to claim 3 or 4 and isolating an glycosylated human Interferon alpha isoform from the culture.

6. A pharmaceutical composition comprising the glycosylated human Interferon alpha isoform according to claim 1 or 2 and a pharmaceutically acceptable carrier.

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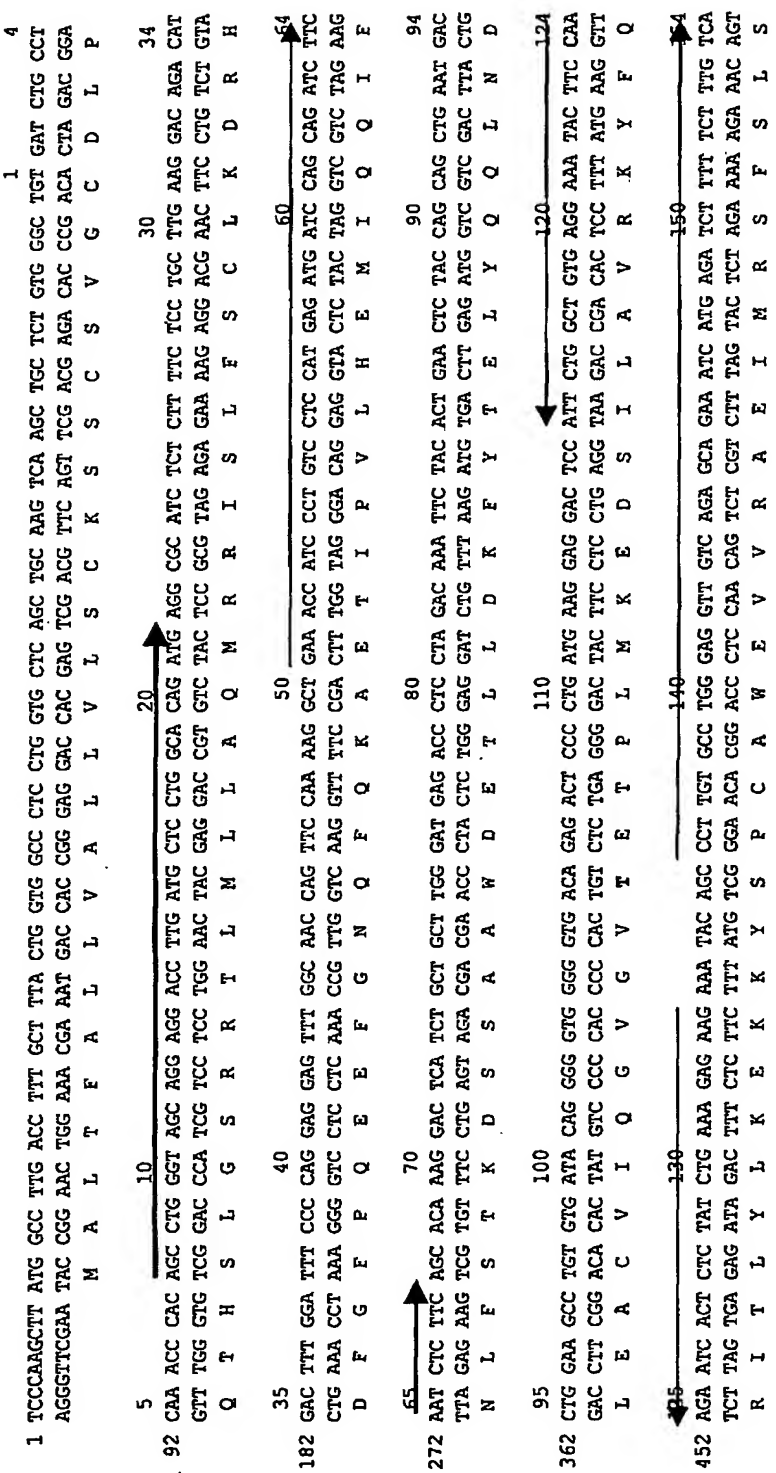
Fig. 1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M A L T F A L L L V A L L V L S C K S S C S V G C D L P
92 CAA ACC CAC AGC CTG GGT AGC AGG AGG ACC TTG ATG CTC CTG GCA CAG ATG AGG CGC ATC TCT CTT TTC TCC TGC TTG AAG GAC AGA CAT
GTT TGG GTG TCG GAC CCA TCG TCC TCC TGG AAC TAC GAG GAC CGT GTC TAC TCC GCG TAG AGA GAA AAG AGG ACG AAC TTC CTG TCT GTA
Q T H S L G S R R T L M L L A Q M R R I S L F S C L K D R H
182 GAC TTT GGA TTT CCC CAG GAG GAG TTT GGC AAC CAG TTC CAA AAG GCT GAA ACC ATC CCT GTC CTC CAT GAG ATG ATC CAG CAG ATC TTC
CTG AAA CCT AAA GGG GTC CTC CTC AAA CCG TTG GTC AAG GTT TTC CGA CTT TGG TAG GGA CAG GAG GTA CTC TAC TAG GTC GTC TAG AAG
D F G F P Q E E F G N Q F Q K A E T I P V L H E M I Q Q Q I F
272 AAT CTC TTC AGC ACA AAG GAC TCA TCT GCT GCT TGG GAT GAG ACC CTC CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG CAG CTG AAT GAC
TTA GAG AAG TCG TGT TTC CTG AGT AGA CGA CGA ACC CTA CTC TGG GAG GAT CTG TTT AAG ATG TGA CTT GAG ATG GTC GTC GAC TTA CTG
N L F S T K D S S A A W D E T L L D K F Y T E L Y Q Q L N D
362 CTG GAA GCC TGT GTG ATA CAG GGG GTG GGG GTG ACA GAG ACT CCC CTG ATG AAG GAG GAC TCC ATT CTG GCT GTG AGG AAA TAC TTC CAA
GAC CTT CGG ACA CAC TAT GTC CCC CAC CCC CAC TGT CTC TGA GGG GAC TAC TTC CTC CTG AGG TAA GAC CGA CAC TCC TTT ATG AAG GTT
L E A C V I Q G V G V T E T P L M K E D S I L A V R K Y F Q
452 AGA ATC ACT CTC TAT CTG AAA GAG AAG AAA TAC AGC CCT TGT GCC TGG GAG GTT GTC AGA GCA GAA ATC ATG AGA TCT TTT TCT TTG TCA
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R I T L Y L K E K K Y S P C A W E V V R A E I M R S F S L S
542 ACA AAC TTG CAA GAA AGT TTA AGA AGT AAG GAA TGA GGATCCCA
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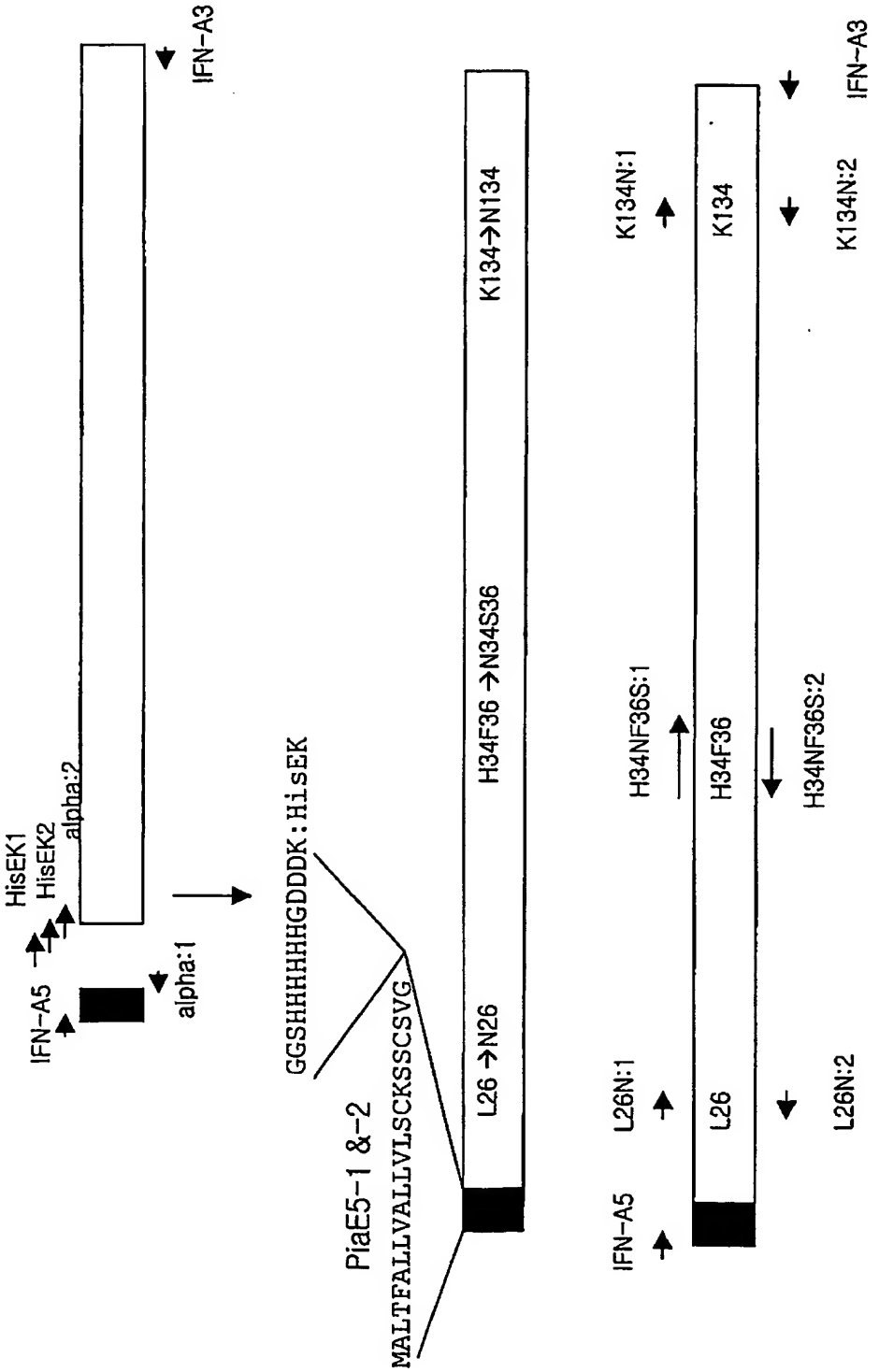
T N L Q E S L R S K E

Fig. 2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H34F36 →N34S36
K134→N134
K134N:1
IFN-A3
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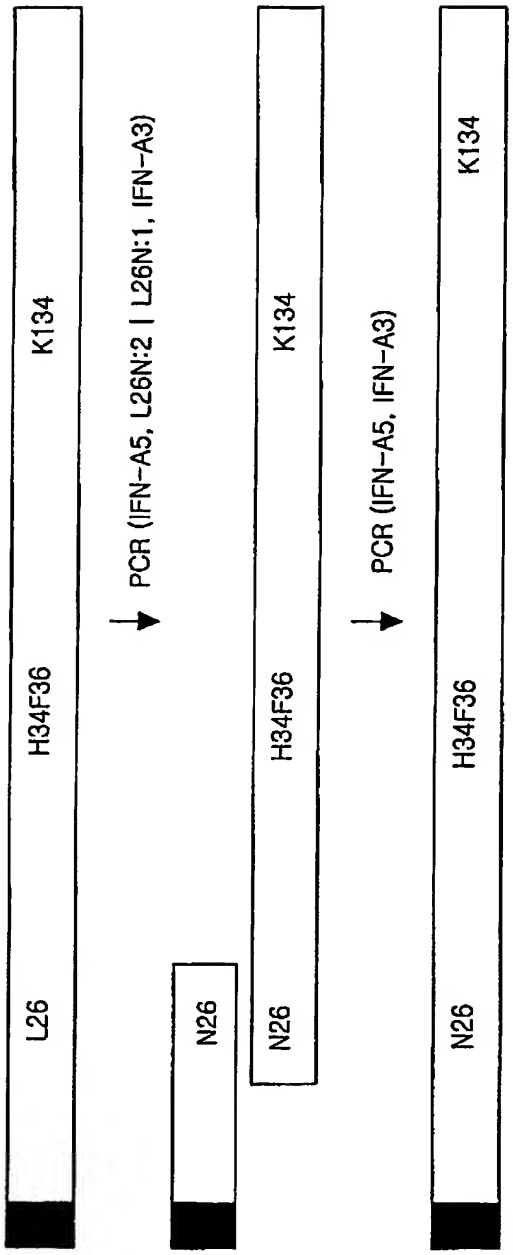
Fig. 3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H34F36
K134
PCR (IFN-A5, L26N:2 I L26N:1, IFN-A3)
N26
H34F36
K134
PCR (IFN-A5, IFN-A3)
N26
H34F36
K134

Fig. 4

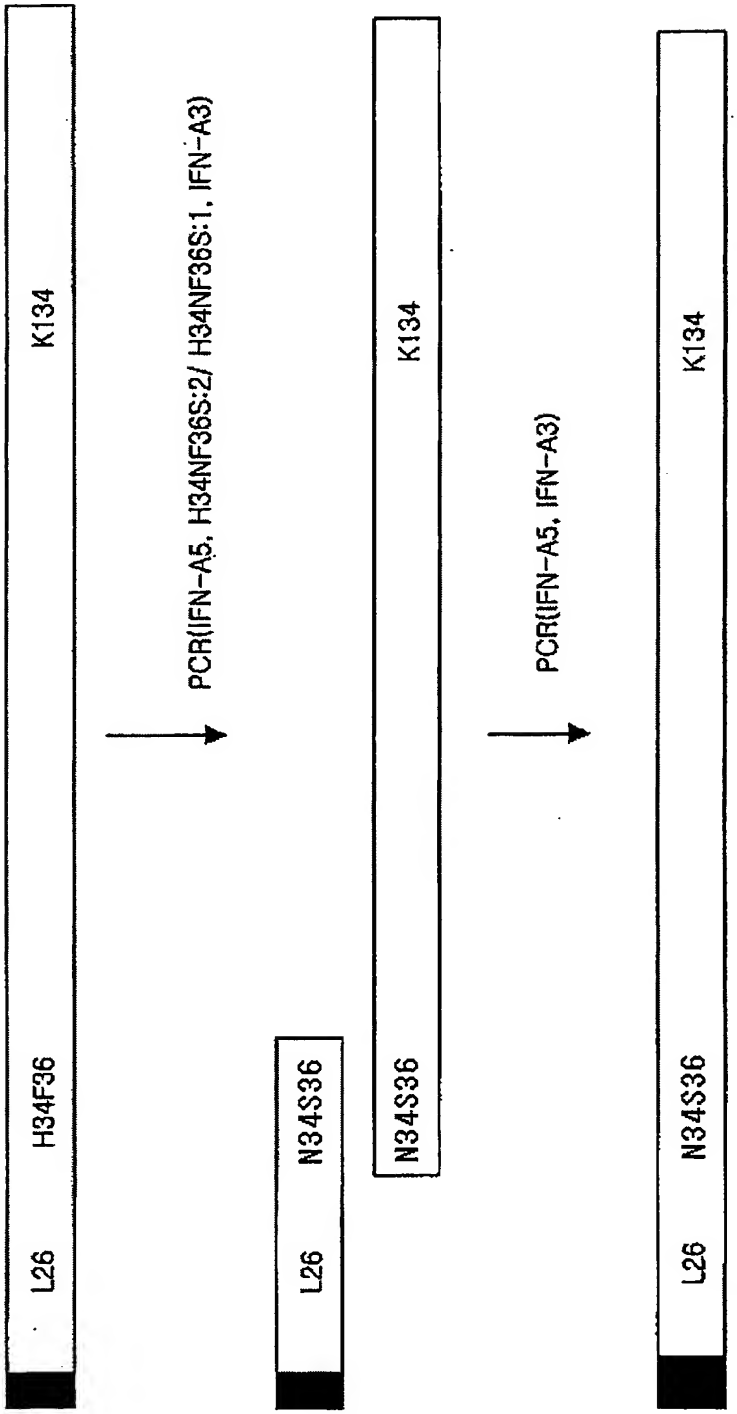
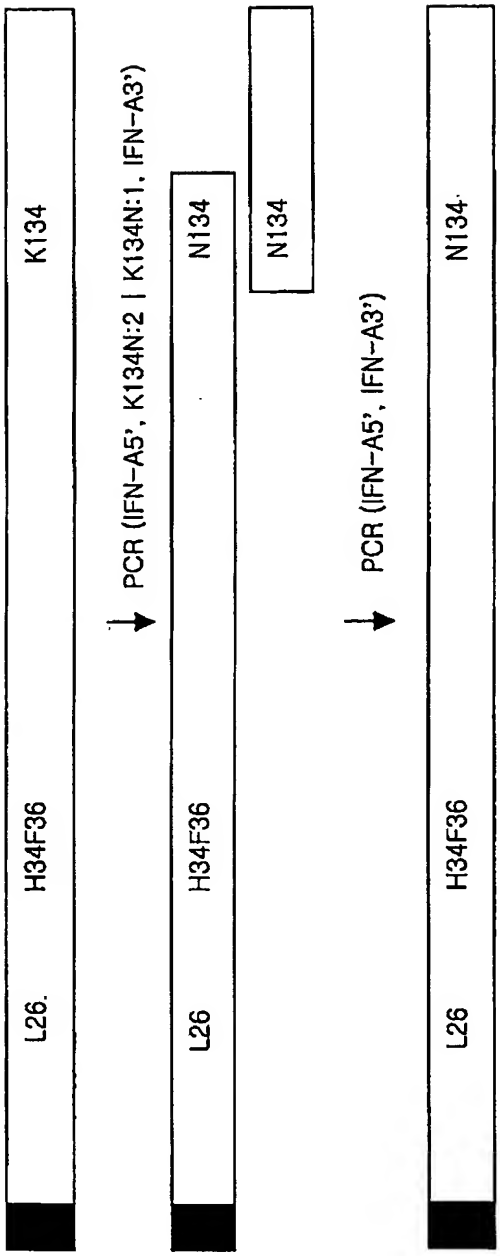

K134
H34F36
L26
PCR(IFN-A5, H34NF36S:2/ H34NF36S:1, IFN-A3)
N34S36
L26
K134
PCR(IFN-A5, IFN-A3)
K134
N34S36
L26

Fig. 5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K134
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N134
N134
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L26
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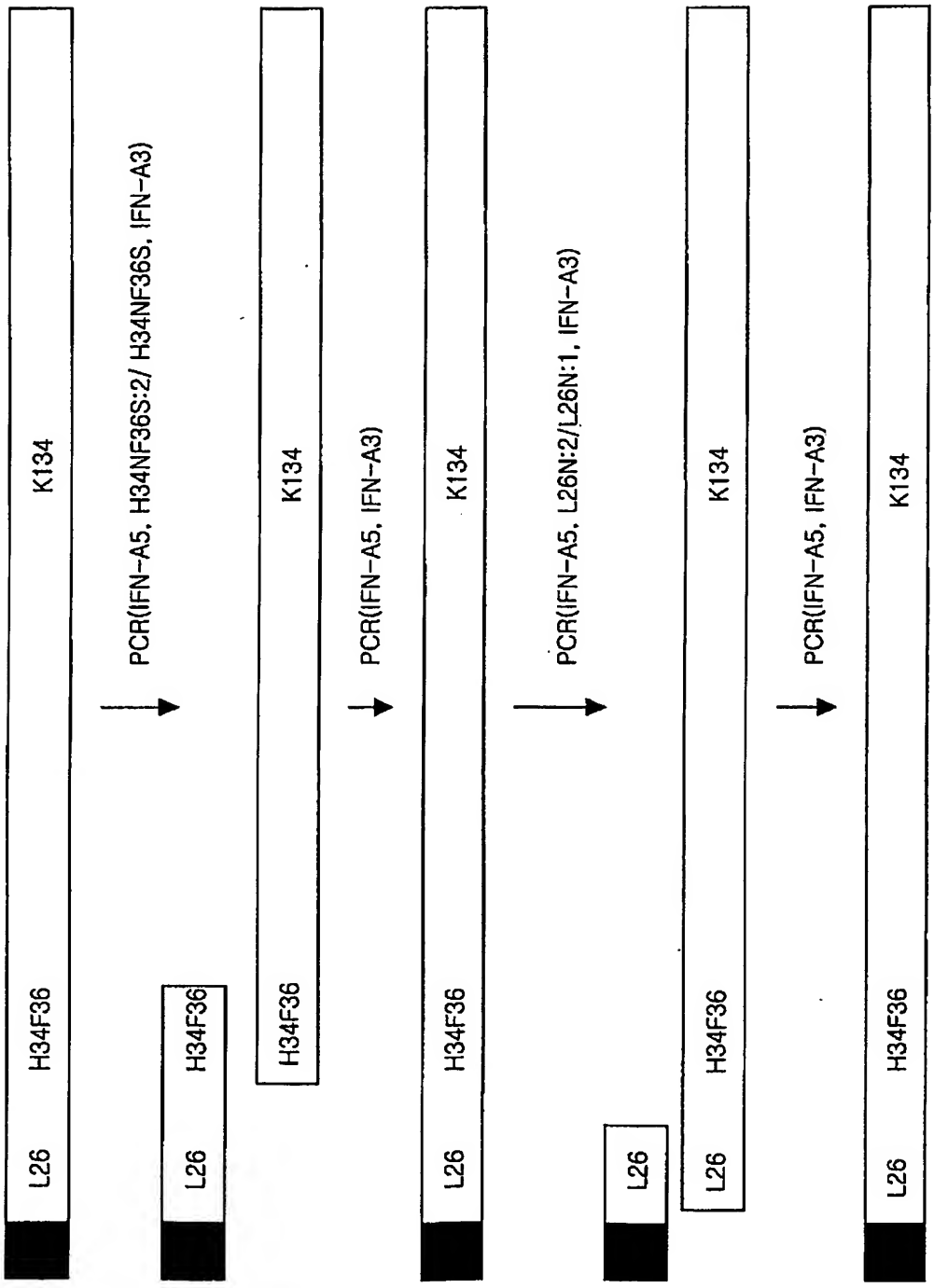
N134

Fig. 6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L26
H34F36
K134
PCR(IFN-A5, L26N:2/L26N:1, IFN-A3)
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K134
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L26
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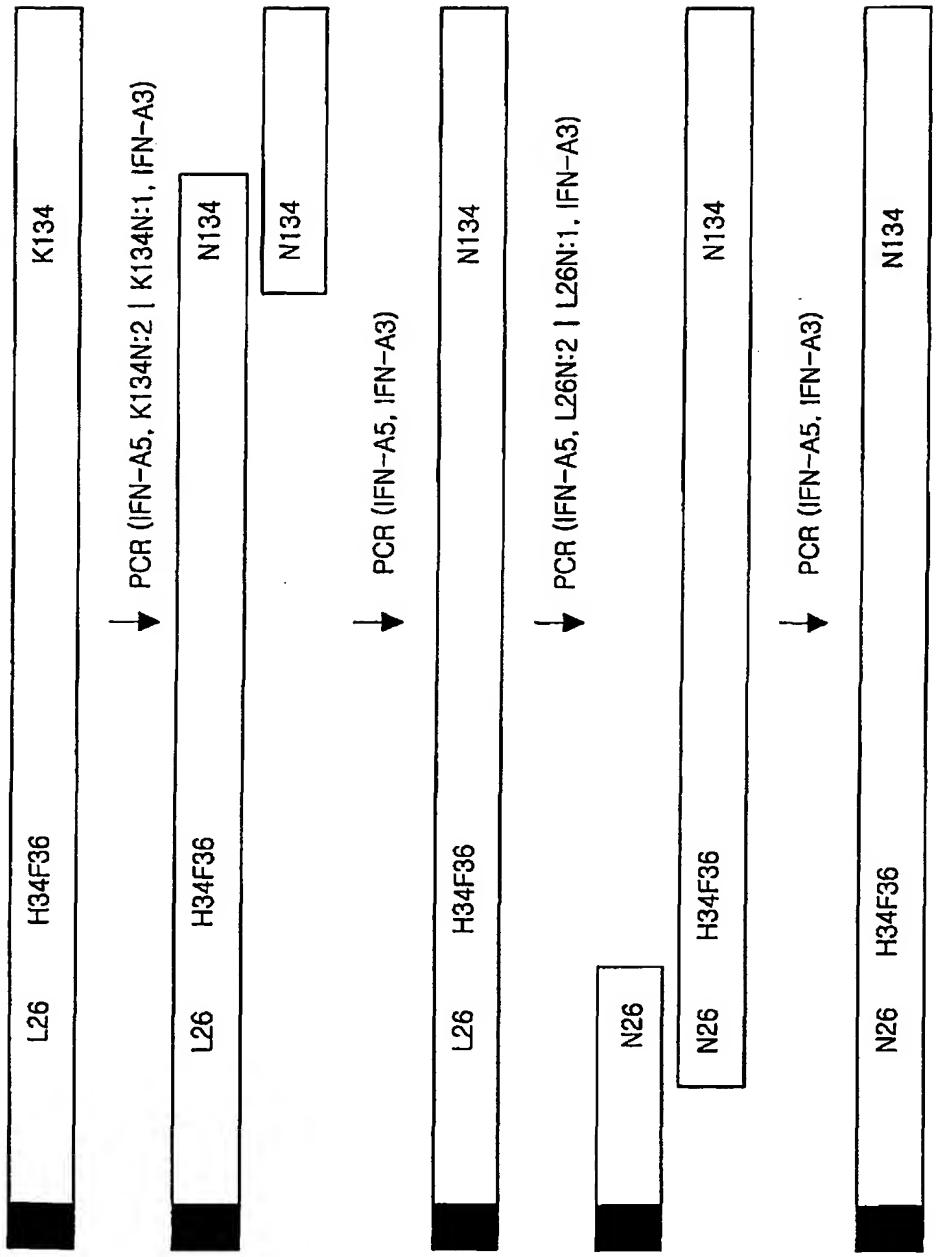
K134

Fig. 7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K134
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N134
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PCR (IFN-A5, IFN-A3)
L26
H34F36
N134
PCR (IFN-A5, L26N:2 | L26N:1, IFN-A3)
N26
N26
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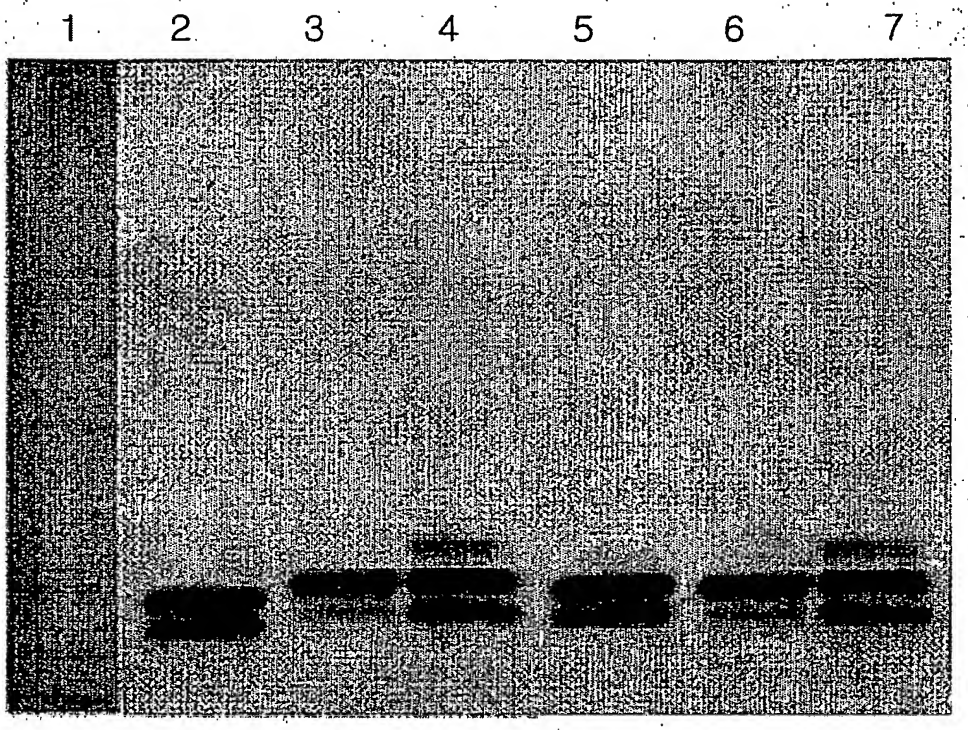
N134
PCR (IFN-A5, IFN-A3)
N26
H34F36
N134

Fig. 8



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2. O-Glycosylated IFN-alpha
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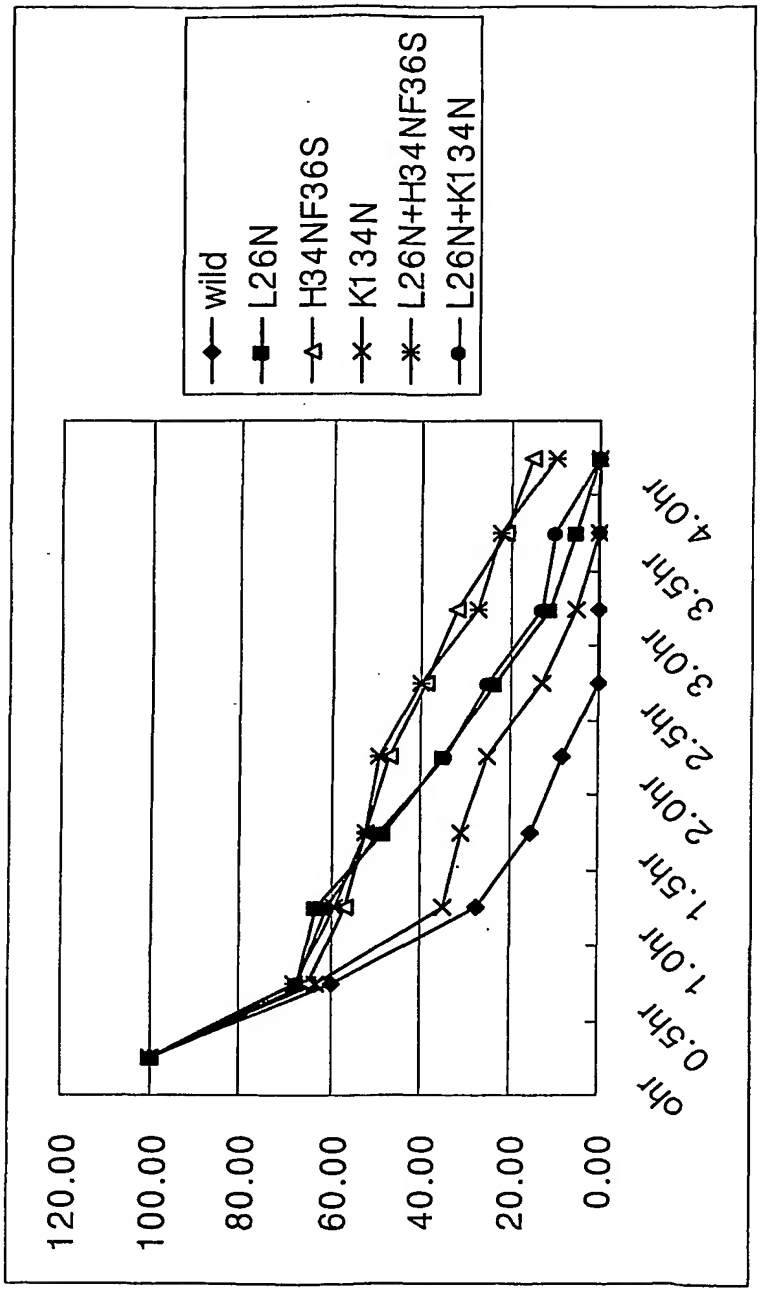
4. L26N/H34NF36S
5. H34NF36S
6. K134N
7. L26N/K134N

Fig. 9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(71) Applicant: **CJ CORP.** [KR/KR]; 500, Namdaemunno 5-ga, Jung-gu, Seoul 100-802 (KR).

(72) Inventors: **LEE, Eun-Jung**; Simgokbon 1-dong, Sosa-gu, Bucheon-si, Gyeonggi-do 422-241 (KR). **PARK, Hyung-Ki**; #601 Sugwang Villart, 1604-3, Seocho 1-dong, Seocho-gu, Seoul 137-071 (KR). **KIM, Hyun-Seok**; #4-1003 Munjeong Siyeong Apt., Munjeong 1-dong, Songpa-gu, Seoul 138-200 (KR). **PARK, Ji-Sook**; #101-801 Siyeong Apt., Junggye-dong, Nowon-gu, Seoul 139-220 (KR). **KIM, Yeon-Hyang**; 100 Sangdo-dong, Dongjak-gu, Seoul 156-030 (KR). **LEE, Hyune-Soo**; #208-202, Sangroksoo Apt., Irwon-dong, Gangnam-gu, Seoul 135-230 (KR). **KOH, Hyung-Kon**; #3-114 Eunma Apt., Daechi 2-dong, Gangnam-gu, Seoul 135-282 (KR). **OH, Myung-Suk**; #374, Dukpyeong-ri, Majang-myeon, Icheon-si, Gyeonggi-do 467-810 (KR).

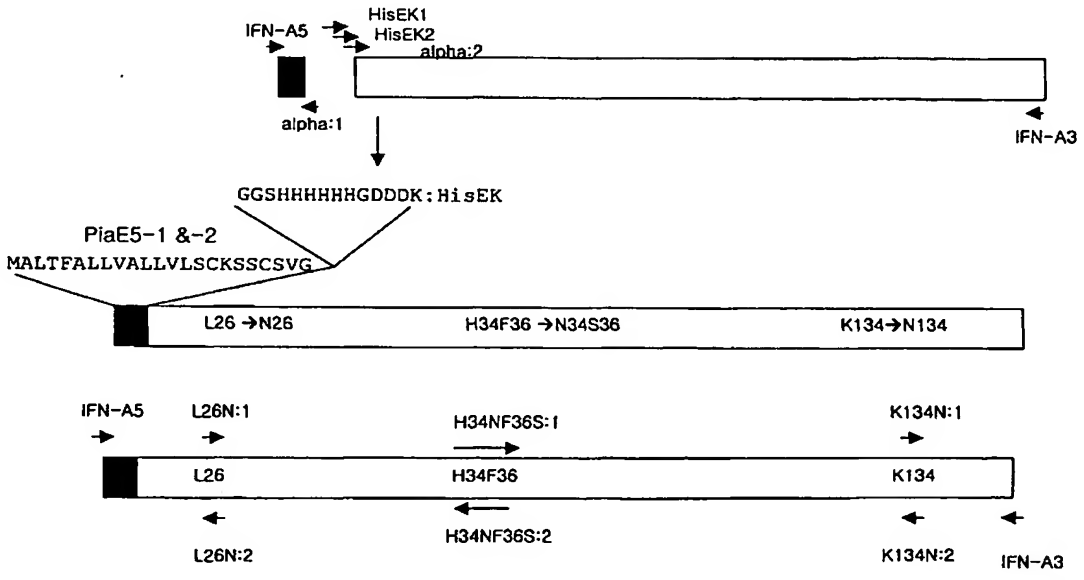
(74) Agent: **SON, Min**; 19th Floor, City Air Tower, 159-9 Samsung-dong, Gangnam-gu, Seoul 135-973 (KR).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: GLYCOSYLATED HUMAN INTERFERON ALPHA ISOFORM



(57) Abstract: The present invention relates to an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at this site and a gene encoding the same, an expression vector comprising the gene, and a method for producing glycosylated human Interferon alpha isoform by transforming or transfecting an eukaryotic cell with the expression vector, culturing the transfected or transformed cell and isolating the glycosylated human Interferon alpha isoform from the culture, the glycosylated human Interferon alpha isoform produced therefrom and a pharmaceutical composition comprising the same.

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European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

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INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/KR03/01765

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C07K 14/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korea Patent and Applications for Inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubMed, CA, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	James D.C., et al. "N-glycosylation of recombinant human interferon-gamma produced in different animal expression systems" Biotechnology (N. Y.)., 1995, Vol. 13(6): pages 592-596, see abstract.	1-6
Y	Sareneva T., et al. "Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma" Biochem. J., 1994, Vol. 303(Pt 3): pages 831-840, see abstract.	1-6
A	Nyberg G.B., et al. "Metabolic effects on recombinant interferon-gamma glycosylation in continuous culture of Chinese hamster ovary cells" Biotechnol. Bioeng., 1999, Vol. 62(3): pages 336-347, see abstract.	1-6
A	Nyman T.A., et al. "Identification of nine interferon-alpha subtypes produced by Sendai virus-induced human peripheral blood leucocytes" Biochem. J., 1998, Vol.329(Pt 2): pages 295-302, see abstract.	1-6

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 DECEMBER 2003 (19.12.2003)	19 DECEMBER 2003 (19.12.2003)
Name and mailing address of the ISA/KR Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea Facsimile No. 82-42-472-7140	Authorized officer PARK, JEONG UNG Telephone No. 82-42-481-8159

Form PCT/ISA/210 (second sheet) (July 1998)

International application No.
PCT/KR2003/001765

Box No. III AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The following person is [X] agent [ ] common representative

and [X] has been appointed earlier and represents the applicant(s) also for international preliminary examination.

[ ] is hereby appointed and any earlier appointment of (an) agent(s)/common representative is hereby revoked.

[ ] is hereby appointed, specifically for the procedure before the International Preliminary Examining Authority, in addition to the agent(s)/common representative appointed earlier.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SON, Min
19th Floor, City Air Tower 159-9 Samsung-dong,
Gangnam-gu, Seoul, 135-973, Republic of Korea

Telephone No.
82-2-2016-7900

Facsimile No.
82-2-2016-7905

Teleprinter No.

Agent's registration No. with the Office

[ ] **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. IV BASIS FOR INTERNATIONAL PRELIMINARY EXAMINATION

Statement concerning amendments:*

1. The applicant wishes the international preliminary examination **to start on the basis of:**

[X] the international application as originally filed

the description [ ] as originally filed
[ ] as amended under Article 34

the claims [ ] as originally filed
[ ] as amended under Article 19 (together with any accompanying statement)
[ ] as amended under Article 34

the drawings [ ] as originally filed
[ ] as amended under Article 34

2. [ ] The applicant wishes any amendment to the claims under Article 19 to be considered as reversed.

3. [ ] The applicant wishes the start of the international preliminary examination **to be postponed** until the expiration of the applicable time limit under Rule 69.1(d).

4. [ ] The applicant expressly wishes the international preliminary examination **to start earlier** than at the expiration of the applicable time limit under Rule 54*bis*.1(a).

* Where no check-box is marked, international preliminary examination will start on the basis of the international application as originally filed or, where a copy of amendments to the claims under Article 19 and/or amendments of the international application under Article 34 are received by the International Preliminary Examining Authority before it has begun to draw up a written opinion or the international preliminary examination report, as so amended.

Language for the purposes of international preliminary examination: English

[X]** which is the language in which the international application was filed.
[ ] which is the language of a translation furnished for the purposes of international search.
[X]** which is the language of publication of the international application.
[ ] which is the language of the translation (to be) furnished for the purposes of international preliminary examination.

** by IPEA

Box No. V ELECTION OF STATES

The filing of this demand constitutes the election of all Contracting States which are designated and are bound by Chapter II of the PCT.

Sheet No. 3

International application No.
PCT/KR2003/001765

Box No. VI CHECK LIST

The demand is accompanied by the following elements, in the language referred to in Box No. IV, for the purposes of international preliminary examination:			For International Preliminary Examining Authority use only: received	not received
1. translation of international application	:	sheets	☐	☐
2. amendments under Article 34	:	sheets	☐	☐
3. copy (or, where required, translation) of amendments under Article 19	:	sheets	☐	☐
4. copy (or, where required, translation) of statement under Article 19	:	sheets	☐	☐
5. letter	:	sheets	☐	☐
6. other *(specify)*	:	sheets	☐	☐

The demand is also accompanied by the item(s) marked below:

1. ☒ fee calculation sheet
2. ☐ original separate power of attorney
3. ☐ original general power of attorney
4. ☒ copy of general power of attorney; reference number, if any:
5. ☐ statement explaining lack of signature
6. ☐ sequence listing in computer readable form
7. ☐ tables in computer readable form related to a sequence listing
8. ☐ other *(specify)*:

by IPEA

Box No. VII SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the demand).

SON, Min

For International Preliminary Examining Authority use only

1. Date of actual receipt of DEMAND: 22 MARCH 2004 (22.03.2004.)

2. Adjusted date of receipt of demand due to CORRECTIONS under Rule 60.1(b):

3. ☐ The date of receipt of the demand is AFTER the expiration of 19 months from the priority date and item 4 or 5, below, does not apply.
   ☐ The applicant has been informed accordingly.
4. ☐ The date of receipt of the demand is WITHIN the time limit of 19 months from the priority date as extended by virtue of Rule 80.5.
5. ☐ Although the date of receipt of the demand is after the expiration of 19 months from the priority date, the delay in arrival is EXCUSED pursuant to Rule 82.
6. ☐ The date of receipt of the demand is AFTER the expiration of the time limit under Rule 54*bis*.1(a) and item 7 or 8, below, does not apply.
7. ☐ The date of receipt of the demand is WITHIN the time limit under Rule 54*bis*.1(a) as extended by virtue of Rule 80.5.
8. ☐ Although the date of receipt of the demand is after the expiration of the time limit under Rule 54*bis*.1(a), the delay in arrival is EXCUSED pursuant to Rule 82.

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Demand received from IPEA on:

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CORRECTED VERSION

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(30) Priority Data:
10-2002-0052365 31 August 2002 (31.08.2002) KR

(71) Applicant: **CJ CORP.** [KR/KR]; 500, Namdaemunno 5-ga, Jung-gu, Seoul 100-802 (KR).

(72) Inventors: **LEE, Eun-Jung**; Simgokbon 1-dong, Sosa-gu, Bucheon-si, Gyeonggi-do 422-241 (KR). **PARK, Hyung-Ki**; #601 Sugwang Villart, 1604-3, Seocho 1-dong, Seocho-gu, Seoul 137-071 (KR). **KIM, Hyun-Seok**; #4-1003 Munjeong Siyeong Apt., Munjeong 1-dong, Songpa-gu, Seoul 138-200 (KR). **PARK, Ji-Sook**; #101-801 Siyeong Apt., Junggye-dong, Nowon-gu, Seoul 139-220 (KR). **KIM, Yeon-Hyang**; 100 Sangdo-dong, Dongjak-gu, Seoul 156-030 (KR). **LEE, Hyune-Soo**; #208-202, Sangroksoo Apt., Irwon-dong, Gangnam-gu, Seoul 135-230 (KR). **KOH, Hyung-Kon**; #3-114 Eunma Apt., Daechi 2-dong, Gangnam-gu, Seoul 135-282 (KR). **OH, Myung-Suk**; #374, Dukpyeong-ri, Majang-myeon, Icheon-si, Gyeonggi-do 467-810 (KR).

(74) Agent: **SON, Min**; 19th Floor, City Air Tower, 159-9 Samsung-dong, Gangnam-gu, Seoul 135-973 (KR).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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see PCT Gazette No. 02/2006 of 12 January 2006, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: GLYCOSYLATED HUMAN INTERFERON ALPHA ISOFORM

(57) Abstract: The present invention relates to an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at this site and a gene encoding the same, an expression vector comprising the gene, and a method for producing glycosylated human Interferon alpha isoform by transforming or transfecting an eukaryotic cell with the expression vector, culturing the transfected or transformed cell and isolating the glycosylated human Interferon alpha isoform from the culture, the glycosylated human Interferon alpha isoform produced therefrom and a pharmaceutical composition comprising the same.

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GLYCOSYLATED HUMAN INTERFERON ALPHA ISOFORM

TECHNICAL FIELD

The present invention relates to a glycosylated human Interferon alpha isoform. More particularly, the present invention relates to a human Interferon alpha isoform having at least one amino acid modified with another amino acid to increase Asn-X-Ser/Thr(N-X-S/T) sequence at a specific region, thereby increasing in vivo stability, and glycosylated human Interferon alpha isoform thereof.

BACKGROUND ART

Interferon were discovered by Isaacs and Lindenmann (Proc. R Soc. Lond[Biol.], 1957, 147, 258-267) in 1957 and has been known to have strong anti-virus effects.

Interferon is classified into a type I Interferon including Interferon-alpha /-beta and a type II Interferon including Interferon gamma. Interferon-alpha is derived from B lymphocyte or macrophage, Interferon-beta is derived from fibroblast and Interferon-gamma is derived from T lymphocyte.

In human, at least 20 kinds of Interferon-alpha genes and pseudo-genes have been identified. Proteins of these Interferon-alphas are shown to have two disulfide bonds (Cys1-Cys98; Cys29-Cys138) in common. Human Interferon-alpha does not contain a N-type glycosylated bond site but wild type mature protein contains an O-type glycosylated bond at Thr of 106th position (Adolf et al., Biochem. J., 276(Pt 2), 511-518, 1991).

Interferon-alpha can be produced in cells of many tissues, however the

productivity is very low. Generally, it is produced largely in leucocytes such as monocyte/macrophage and B lymphocyte. Here, the proportion of subtypes in produced Interferons varies depending on produced cell types and production conditions. It has been known that the production of Interferons is induced by virus infection. Further, bacteria, mycoplasma, protozoa and the like may induce the production of Interferon and particularly, lipopolysaccharide (LPS) of gram negative bacteria is a strong Interferon inducing agent.

mRNA of Interferon-alpha is continuously produced even in tissues of a normal human (Tovey et al., Proc Natl Acad Sci USA, 1987, vol. 84, 5038-5042). It is believed that this Interferon is an autocrine Interferon playing a important role in growth and differentiation of cell.

The working mechanism in vivo of Interferon is not known yet. According to the report by Branca and Baglioni (Nature, 294, 768-770, 1981), it was shown that Interferon-alpha and –beta bind to the same receptor in human lymphoblastoid cell.

When virus infection takes place in vivo, Interferon is produced and the produced Interferon induced proteins, which perform Interferon's functions. Representative examples of such proteins include 2'- 5'- oligoadenylate synthetase and protein kinase phosphorylation of elF2 (elongation factor2) which is a factor involved in initiation of peptide chain synthesis. The two enzymes are activated double-stranded RNA (Lengyel P., Annu. Rev. Biochem., 51, 251-282, 1982; PestKa et. al., Annu. Rev. Biochem., 56, 727-777, 1982; De Maeyer and De Maeyer-Guignard J., Interferons and other regulatory cytokines, Wiley, New York).

Interferon is clinically applied in treatment of chronic active hepatitis B, acute viral encephalitides, nasopharyngeal carcinoma and the like.

Since most of bioactive proteins used as medicaments shows low stability in living bodies, patients who need the bioactive proteins should frequently receive excessive amounts to maintain a certain level of the proteins so that they can work. Therefore, patients suffers pain and inconvenience and it is desired to produce a bioactive protein having in vivo stability enhanced to alleviate the suffering of these patients.

International Patent Application Publication No. WO 98/48840 discloses a preparation of Interferon alpha conjugated with polyethyleneglycol as a polymer to increase invito stability of bioactive proteins or US PAT No. 6,399,103 discloses a preparation of a medicament by microcapsulation of human growth hormone. However, these methods accompany complicated processes involving primary production of a protein from a microorganism, followed by purification, and subsequent addition reactions. Also, a cross-linking may takes place at a undesired site and homogeneity of the final product may cause a problem. Another approach is a method using glycosylation. Cell surface proteins and secretion proteins produced by eukaryotic cells can be modified by glycosylation. It is known that the glycosylation can affect not only physical properties of a protein but also stability and functions of a protein in living bodies.

DISCLOSURE OF THE INVENTION

Therefore, it is an object of the present invention to readily produce a target protein from a cell line by glycosylation at human Interferon alpha using gene recombinant

technology and prepare a protein which increased in vivo stability.

In one aspect, the present invention provides an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

In another aspect, the present invention provides a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In a further aspect, the present invention provides an expression vector comprising a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In yet further aspect, the present invention provides a transformed or transfected host cell with an expression vector comprising a gene encoding an amino acid-modified

human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In yet further aspect, the present invention provides a method for preparing glycosylated human Interferon alpha comprising culturing a transformed or transfected host cell with an expression vector comprising a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site in a suitable medium under suitable conditions to isolate glycosylated human Interferon alpha isoform.

In yet further aspect, the present invention provides glycosylated human Interferon alpha isoform obtainable by additional glycosylation of an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

In yet further aspect, the present invention provides a pharmaceutical composition comprising a glycosylated human Interferon alpha isoform obtainable by additional glycosylation of an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site and a pharmaceutically acceptable carrier.

In yet another aspect, the present invention provides a synthetic oligodeoxynucleotide used as a primer for production of a glycosylation site in human Interferon alpha protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings in which:

Fig. 1 shows the sequence of human Interferon alpha gene and protein. The arrows or straight lines over the sequence represent regions of a helical configuration in the three-dimensional structure of human Interferon alpha protein, the arrow direction represents the direction of the helix according to the order of the amino acid sequence.

Arginine, 23rd amino acid of mature Interferon alpha, has a DNA sequence different from that of the known to the art but encodes the same amino acid. Before the modification, 106th threonine of the mature Interferon alpha protein is a site where glycosylation (O-type) takes place upon production of a human-derived or eukaryotic cell;

Fig. 2 shows a site in the protein structure of human Interferon alpha where amino acid modification of glycosylation according to the present invention takes place, in which the site contains the pre-sequence, 6 histidines as amino acids capable of bonding to a metal ion for readiness of purification and an enterokinase digested site (4 aspartic acids and subsequent lysine sequence);

Fig. 3 is a schematic diagram to show the method for modifying leucine, 26th amino acid with asparagine;

Fig. 4 is a schematic diagram showing the method for modifying histidine, 34th amino acid and phenylalanine, 36th amino acid with asparagine and serine, respectively;

Fig. 5 is a schematic diagram showing the method for modifying lysine, 134th amino acid of wild-type Interferon alpha with asparagine;

Fig. 6 is a schematic diagram showing the method for simultanenously modifying 26th leucine, 34th phenylalanine and 36th phenylalanine of wild-type Interferon alpha with asparagine, asparagine and serine, respectively;

Fig. 7 is a schematic diagram showing the method for simultaneously modifying 26th leucine and 134th lysine with asparagine, respectively;

Fig. 8 shows the result of western blot on human Interferon alpha derivatives.

The primary antibody is a monoclonal antibody against for human Interferon alpha and the secondary antibody is an antibody of rabbit antibody against mouse immunoglobuline bonded to HRP enzyme. Here, 1 represents a marker, 2 represents O-glycosylated IFN-alpha, 3 represents L26N mutant, 4 represents L26N/H34NF36S mutant, 5 represents H34NF36S mutant, 6 represents K134N mutant and 7 represents L26N/K134N mutant; and

Fig. 9 is a graph showing the residual concentration of human Interferon alpha derivatives in mouse according to elapsed time.

BEST MODES FOR CARRYING OUT THE INVENTION

The term "isoform of human Interferon alpha" used herein refers to an analogue or mutant having one or more of inherent amino acid sequence residue of wild-type human Interferon alpha modified with another amino acid while maintaining its inherent activities.

The three letters (single letter) of amino acids used herein mean the following acids according to standard abbreviation regulation in the biochemistry field:

Ala(A): alanine; Asx(B): asparagine or aspartic acid; Cys(C): cystein;

Asp(D): aspartic acid; Glu(E): glutamic acid; Phe(F): phenylalanine;

Gly(G): glycine; His(H): histidine; Ile(I): isoleucine; Lys(K): lysine; Leu(L): leucine; Met(M): methionine; Asn(N): asparagine; Pro(P): proline;

Gln(Q): glutamine; Arg(R): arginine; Ser(S): serine; Thr(T): threonine; Val(V): valine; Trp(W): tryptopan; Tyr(Y): tyrosine; Glx(Z): glutamine or glutamic acid.

"(amino acid single letter)(amino acid position)(amino acid single letter)" used herein refers that the former amino acid at the corresponding amino acid position of human Interferon alpha is substituted with the latter amino acid. For example, L26N indicates that leucine corresponding No. 26 of whild-type human Interferon alpha is substituted with asparagine.

In the present specification, a primer for production of glycosylation site is expressed as "(amino acid single letter)(amino acid position)(amino acid single letter) 1 or 2", in which 1 is a primer complementary to a single strand template proceeding 5'→3' direction in a double strand template and 2 is a primer complementary to a single strand template proceeding 3'→5' direction in a double strand template.

Secretion proteins produced by eukaryotic cells as a host cell may be modified by at least one oligosaccharide. It was known that such modification called glycosylation may enormously affect to physical properties of the proteins and be critical in stability, secretion and location in a cell of the proteins. Proper glycosylation may be necessary for biological activity. In practice, when a gene derived from an eukaryotic cell is expressed in bacterium lacking a intracellular process to glycosylate a protein, a protein with deteriorated activity due to the lack of glycosylation is produced.

The glycosylation takes place at a certain position depending on a polypeptide backbone, typically including two types. One is O-type glycosylation which involves binding of oligosaccharide to –OH group of serine or threonine residue and the other is N-type glycosylation which involves binding of oligosaccharide to –NH group of asparagine residue. Particularly, the N-type glycosylation takes place in case having a specific amino acid sequence and the sequence is known as Asn-X-Ser/Thr(N-X-S/T), in which X may be any amino acid except for proline. The N-linked oligosaccharide and the O-linked oligosaccharide have different structures and residues found in each type are also different from each other. For example, in the O-linked saccharide residue, N-acetylgalactosamine is always bonded to serine or threonine while in the N-linked saccharide residue, N-acetylglucosamine is always bonded to asparagines. The O-linked oligosaccharide generally comprises 4 or less of saccharide residues while the N-linked oligosaccharide always contains N-acetylglucosamine and mannose and comprises at least 5 saccharide residues.

The present invention relates to an amino acid-modified human Interferon alpha isoform to increase in vivo stability of a protein comprising at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at a specific site so that glycosylation takes place at the site.

The present inventors have discovered that glycosylation by amino acid modification may be induced at any region except for the helical region in the amino acid sequence of human Interferon alpha protein.

In one embodiment, the present invention is directed to an amino acid-modified

human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

In a preferred embodiment, the present invention is directed to an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52); and

-Lys134-Ser137(Lys134-Tyr-Ser137).

In a more preferred embodiment, the present invention is directed to an amino

acid-modified human Interferon alpha isoform having 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or having all of these modifications.

The present invention comprising modifying at least one nucleotide so that N-type glycosylation may occur on a DNA sequence encoding human Interferon alpha to have an additional glycosylation site, introducing the DNA glycosylation to an eukaryotic cell carrying out the glycosylation, followed by expression so that the additional glycosylation naturally occurs. The additionally glycosylated human interferon alpha according to the present invention is achieved by modifying the DNA sequence so that Asn-X-Ser/Thr(N-X-S/T) sequence is increased.

In one embodiment, the present invention is directed to a gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

In a preferred embodiment, the present invention is directed to a gene encoding an amino acid-modified human Interferon alpha isoform having at least one amino acid modified with another amino acid so that the Asn-X-Ser/Thr(N-X-S/T) sequence is increased at the following amino acid residue positions:

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52); and

-Lys134-Ser137(Lys134-Tyr-Ser137).

In a more preferred embodiment, the present invention is directed to directed to a gene encoding an amino acid-modified human Interferon alpha isoform having 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or having all of these modifications.

In one embodiment of the present invention, the gene encoding human Interferon alpha is obtained from human Interferon alpha-producing strain for animal cell expression. For gene cloning and separation, methods known to the art may be used.

The human Interferon alpha gene obtained from the above may be modified in at least one selected codon. In the present specification, modification may be defined as substitution of one or more codon(s) on a gene encoding human Interferon alpha to make a change in the amino acid sequence of human Interferon alpha. More particularly, it refers to substitution of at least one amino acid with another amino acid so that the Asn-X-

Ser/Thr(N-X-S/T) sequence for additional N-type glycosylation is formed on the amino acid sequence of human Interferon alpha. For example, in Example 3 of the present invention, when 26th leucine is substituted with asparagine, since 28th amino acid is serine, the Asn-X-Ser/Thr(N-X-S/T) sequence is formed, whereby an additional N-type glycosylation may take place. Also, when 34th histidine and 36th phenylalanine are substituted with asparagine and serine, respectively, the Asn-X-Ser/Thr(N-X-S/T) sequence is formed, whereby an additional N-type glycosylation may take place. Further, when 134th lysine is substituted with asparagine, since 136th amino acid is serine, the Asn-X-Ser/Thr(N-X-S/T) sequence is formed, whereby an additional N-type glycosylation may take place.

In one embodiment, a synthetic oligonucleotide comprising a codon encoding a desired amino acid modification in human Interferon alpha is constructed. Typically, an oligonucleotide having a length of about 25 nucleotides is used. Though an oligonucleotide with a more shorten length can be employed, the optimal oligonucleotide is to have 12 to 15 nucleotides complementary to a template at both sides of the nucleotides encoding the modification. Such oligonucleotide may be sufficiently hybridized to the template DNA. The synthetic oligonucleotides used for production of an additional glycosylation site in the present invention are shown in Table 2. These oligonucleotide can be synthesized by technologies known to the art.

In one embodiment of the present invention, there is provided a human Interferon alpha isoform DNA with one amino acid modified. PCR is conducted using human Interferon alpha DNA is used as a template and a synthetic oligonucleotide encoding a

modification as a primer. In the heating step of PCR, the double stranded template is separated and to each of the single strand template, a complementary primer is hybridized. DNA polymerase bonds nucleotides complementary to the template from –OH group of the primer encoding the modification in 5′ → 3′ direction. Consequently, the second strand contains the primer encoding the modification and thus encodes the desired modification on a gene. The second strand serves as a template DNA in the repeated replication steps of PCR and the gene encoding the modification will be continuously amplified. For example, in Example 3 of the present invention, in order to modify leucine, the 26th amino acid residue with asparagine, PCR is conducted using wild-type Interferon alpha DNA as a template and primer pairs of IFN-A5' and L26N2, and L26N1 and IFN-A3'. As a result, two DNA segments, in which 26th amino acid position is changed to a codon corresponding to asparagine instead of leucine, are obtained. Then, secondary PCR is conducted using the two DNA segments thus obtained and IFN-A5' and IFN-A3' as a primer pair to obtain a modified gene of IFN-alpha-L26N, in which 26th amino acid is modified with asparagine instead of leucine so that a glycosylation may occur.

In another embodiment of the present invention, there is provided a human Interferon alpha isoform comprising two or more amino acid modifications. A mutant having two or more amino acids modified is constructed by various methods. When the two or more amino acids to be modified are adjacent to each other on a polypeptide, they can be simultaneously modified using an oligonucleotide having all the amino acid modifications encoded. Therefore, the construction of the mutant is the same with the method for construction of a human Interferon alpha gene with one nucleotide modified

except that an oligonucleotide having two or more amino acid modifications as a primer. However, when the two or more amino acids are far from each other on a polypeptide (spaced by 10 or more amino acids), it is impossible to construct an oligonucleotide having all the desired modifications encoded.

Instead, other methods should be introduced. The fist method is to construct individual oligonucleotides containing each amino acid modification. If the oligonucleotides are simultaneously annealed to a single strand template DNA, the second strand DNA synthesized from the template will encode all the desired amino acid modifications. Another method in the present invention includes two or more times mutagenesis to produce such an isoform. In the first mutagenesis, wild-type DNA is used as a template and an oligonucleotide containing the first desired amino acid modification is annealed to the template to form a heterogeneous DNA (heteroduplex). In the second mutagenesis, the modified DNA, prepared in the first mutagenesis, is used as a template. Thus, this template already contain at least one modification. To this template, an oligonucleotide containing an additional amino acid modification is annealed and the resulting DNA have all the modifications of the first and second mutagenesis encoded.

The resulting DNA can be used as a template in the third mutagenesis. In summary, the foregoing method for modifying two or more nucleotides is to repeat a method for modifying one nucleotide several times. For example, in Example 3 of the present invention, to modify leucine, 26th amino acid of wild-type Interferon alpha protein with asparagine and 134th amino acid, lysine with asparagine at the same time, firstly 134th position is modified and a modification of 26th amino acid is conducted using the

previously modified DNA as a template. As a result, a human Interferon alpha gene having the two residues modified is obtained.

The DNA sequences encoding the human Interferon alpha isoforms according to the present invention can be synthesized by any standard method known to the art, for example using an automatic DNA synthesizer (ex. Biosearch, Applied Biosystem™).

The glycosylated isoform according to the present invention is typically produced by (a) inserting the DNA sequence encoding human Interferon alpha isoform to a vector having one or more expression control sequence operatively linked to the DNA sequence to control its expression, (b) transforming or transfecting a host with the resulting recombinant expression vector, (c) culturing the transformed or transfacted cell in a proper medium and condition to express the human Interferon alpha isoform DNA sequence, followed by isolation of the glycosylated human Interferon alpha isoform.

In connection with this, the present invention provides a host cell transformed or transfected with the recombinant expression vector containing the DNA sequence encoding the human Interferon alpha isoform.

Of course, it should be understood that all the vectors and expression control sequences do not equally play their functions to express the DNA sequence according to the present invention. Similarly, all the host cells do not equally play their functions for the same expression system. However, those skilled in the art may properly select a vector, expression control sequence and host cell without departing the scope of the present invention while not bearing excessive experiments. For example, in selection of a vector, a host cell must be considered. This is because the vector should be replicated therein.

Also, the replication number and ability to control the replication number of a vector and expression of other proteins encoded by the vector, for example antibiotic marker should be considered. In selection of an expression control sequence, various factors should be considered. For example, relative strength of the sequence, controllability and compatibility with the DNA sequence of the present invention, particularly with respect to a possible two-dimensional structure should be considered. Also, in selection of a host, compatibility with a selected vector, toxicity, secretion properties and ability to correctly fold a polypeptide of the product encoded by the nucleotide sequence, fermentation or cultivation requirements and conditions and readiness of purification of the product encoded by the nucleotide sequence.

The term "vector" used herein refers to a DNA molecule as a carrier capable of stably carrying a foreign gene into a host cell. In order to be a useful vector, a vector can be replicated, has a means to be introduced into a host cell and to detect its own presence.

The term "recombinant expression vector" refers to a cyclic DNA molecule, in which a foreign gene is operably linked to a vector so that the gene can be expressed in a host cell. The recombinant expression vector can be produced as several copies and heterogeneous DNA inserted therein. As well-known to the art, in order to increase expression level of a transfected gene in a host cell, the gene should be operably linked to open frame expression control sequence which can work in a selected expression host. Preferably, the gene is contained in an expression vector comprising a selection marker and replication origin. When an expression host is a eukaryotic cell, the expression vector should further comprise an expression marker useful in the eukaryotic expression host cell.

Various expression vectors can be used to express the DNA sequence encoding the human Interferon alpha isoform. Preferably, an expression vector suitable for an eukaryotic host cell since glycosylation takes place on the human Interferon alpha isoform.

Examples of expression vectors useful for eukaryotic host cells include expression control sequences derived from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific examples of the vectors include pCDNA3.1(+)\Hyg(Invitrogen, Carlsbad, Calif., USA) and pCI-neo(Stratagen, La Jolla, Calif., USA). Expression vectors useful for yeast cells include 2 μ plasmid and derivatives thereof, POT1 vector(U.S. Pat. No. 4,931,373) and pPICZ A, B, or C(Invitrogen). Expression vectors useful for insect cells include pVL·941, pBluebac 4.5 and pMelbac(Invitrogen).

"Expression control sequence" refers to nucleic acid sequences necessary or beneficial to polypeptide expression. Respective expression control sequences can be a native or foreign on a nucleic acid encoding a polypeptide. Examples of the control sequence include, but are not limited thereto, leader sequence, polyadenylated sequence, propeptide sequence, promoter, enhancer or upstream activation sequence, signal peptide sequence and transcription termination factor. An expression control sequence contains a promoter.

In order to express the DNA sequence of the present invention, various expression control sequences can be used as a vector. Examples of expression control sequence suitable to instruct expression in mammal cells include early and late promoters of SV40 and adenovirus, MT-1(metallothioneine gene) promoter, human cytomegalovirus early

gene(CMV), Raus sarcoma virus (RSV) promoter and human Ubiquitine C (UbC) promoter. In order to further improve expression in mammal cells, synthetic intron can be inserted into a non-transcription region of a nucleotide sequence encoding a polypeptide.

Examples of expression control sequences suitable to instruct expression in insect cells include polyhedrin promoter, P10 promoter, baculovirus 39K delayed-early gene promoter and SV40 polyadenylation sequence. Examples of expression control sequences usable in yeast cells include a promoter of α-mating system, yeast triose phosphate isomerization enzyme (TPI) promoter and ADH2-4c promoter. Examples of expression control sequences suitable to instruct expression in fungus cells include ADH3 promoter and termination factor.

Other usable component of a vector used in practicing the present invention is a signal peptide. This sequence is typically located at 5' of a gene encoding a protein and is thus, transcripted to amino terminus of the protein. Presence or absence of a signal peptide varies depending on an expression host cell used in production of a polypeptide to be expressed (according to whether the polypeptide to be expressed is intracell or extracell polypeptide) and preference of recovering secreted products. The signal peptide exists when a polypeptide is secreted from a expressed cell. If the signal peptide exists, it should be recognized by a cell selected for expression of a polypeptide. The signal peptide peptide can be homologus to a polypeptide (typically associated with the polypeptide) or heterologus to a polypeptide (derived from one other than the polypeptide) and can be homologus or heterologus to a host cell.

A nucleic acid is "operably linked" to another nucleic acid when they are arranged

in a functional relationship. This means that an appropriate molecule (for example, a transcription activator) binds to a regulatory sequence(s), a gene or a regulatory sequence(s) linked in such a way that the expression of the gene is modulated. For example, when a pre-sequence or secretory leader participates in secretion of a mature protein, they are operably linked to the promoter. When a promoter affects transcription of a coding sequence, the promoter is operably linked to the coding sequence. When a ribosomal binding site is located at a place capable of reading a coding sequence, the ribosomal binding site is operably linked to the coding sequence. Generally "operably linked" means that to contact with a linked DNA and a secretory leader and to be in a reading frame. However, the enhancer does not need to contact. The linkage of these sequences are effected by ligation (linkage) in a convenient restriction enzyme site. If such a site does not exist, a conventionally synthesized oligonucleotide adaptor or linker may be used.

Construction of a suitable vector comprising a gene encoding the human Interferon alpha isoform and the foregoing components (i.e. a control sequence) can be performed using a basic recombinant technology. In order to prepare a desired vector, respective DNA segments are firstly digested with restriction enzymes and then ligated to each other considering a particular order and orientation.

DNA can be digested using a particular restriction enzyme in a proper buffer.

Typically, about 0.2~1ug of a plasmid or a DNA segment is used along with about 1 to 2 units of a needed restriction enzyme in about 20 μl of buffer. A proper buffer, DNA level, incubation time and temperature are specified by a manufacturer of the restriction enzyme. Typically, it is suitable to incubate for about 1 to 2 hours at 37℃,

though some enzymes need a higher temperature. After incubation, enzymes and other impurities can be removed by extraction the digestion solution with a mixture of phenol and chloroform and DNA can be recovered from the aqueous layer by precipitation with ethanol. Here, ends of the DNA segments are compatible with each other so that the DNA segments can form a functional vector.

The digested DNA segments are classified and selected according to their sizes by electrophoresis. DNA can be electrophoresed through agarose or polyacrylamide matrix. Selection of the matrix can be determined by a size of the DNA segment to be isolated. After electrophoresis, DNA is extracted from the matrix by electroelution. When a low-melting agarose is used, agarose is melted and DNA is extrated therefrom.

The DNA segments to be ligated should be added to the solution in an equal molar amount. The solution contains ATP, ligase buffer, ligases such as about 10 units of T4 ligase per DNA 0.5ug. In order ligate a DNA segment to a vector, the vector should be linearlized through digestion with a suitable restriction enzyme. The linearlized vector is treated with alkaline phosphatase or calf intestinal alkaline phosphatase. The treatment with phosphorylase inhibits self-ligation of a vector during the ligation step. The recombinat expression vector prepared by the above-described method is then used to transform or transfect a host cell.

In selection of a host cell, a host cell having a high DNA introduction efficiency and showing a high expression efficiency of the introduced DNA. Particualrly, in the present invention, eukaryotic host cells to perform glycosylation to the human Interferon alpha isoform is used. Suitable examples of yeast host cells include Saccharomyces and

Hansenula strains. Suitable examples of fungus host cells include Tricoderma, Fusarium and Aspergillus strains. Suitable examples of insect host cell include Lepidoptora cell lines such as Sf9 or Sf21. Suitable examples of mammal host cells include CHO cell line, COS cell lines such as COS 1, COS 7, BHK cell lines and animal cells such as mouse cells, tissue cultured plant cells and human cells.

Polynucleotide can be introduced to a host cell by methods described in basic experiment manuals such as [Davis et al., Basic Methods in Molecular Biology(1986)] and [Sambrook et al., (1989) Molecular Cloning 2nd Edition]. Preferred methods for introducing a polynucleotide to a host cell include, for example, calcium phosphate transfection, DAEA-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction or infection.

In the production method of the present invention, host cells are cultured in a nutrient medium suitable for polypeptide production using a known technoloty. For example, cells can be cultured in a suitable medium in a fermenter for laboratory or industry under conditions acceptable for expression and/or secretion of a polypeptide by small-scale or large scale fermentation, shake flask culture. The cultivation is conducted in a proper nutrient medium comprising carbon, nitrogen supply source and inorganic salts using a known technology. The medium is well known to those skilled in the art and is commercially available or can be produced. When a peptide is directly secreted to a nutrient medium, the polypeptide can be directly isolated from the medium. When a polypeptide is not secreted, it can be isolated from cell lysate

A polypeptide can be isolated by a method known to the art. For example, it can be isolated from a nutrient medium by traditional methods including, but not limited thereto, centrifugation, filtration, extraction, spray drying, evaporation or precipitation. Further, a polypeptide can be purified by various methods known to the public including chromatograph (ex. Ion exchange, affinity, hydrophilic, hydrophobic, size-exclusion), electrophoresis, fractional solubility (ex. Ammonium sulfate precipitation), SDS-PAGE or extraction.

The present invention provides a glycosylated human Interferon alpha isoform with an additional glycosylation obtainable through the above described procedure. In the present specification, the glycosylated human Interferon alpha isoform can be defined as an expression product obtained by introducing a human Interferon alpha gene which is modified to increase the Asn-X-Ser/Thr(N-X-S/T) sequence into an eukaryotic host cell, follwed by expression so that glycosylation can spontaneously occur. That is, it refers to a heterogenous molecular formed by covalent bonding of sugar residues to asparagine –NH group of Asn-X-Ser/Thr(N-X-S/T), an additional glycosylation site of the human Interferon alpha isoform.

The present invention provides a pharmaceutical composition comprising glycosylated human Interferon alpha isoform with an additional glycosylation and a pharmaceutically acceptable carrier. A therapeutic preparation of the glycosylated human Interferon alpha isoform for therapeutic administration can be formulated into lyophilized cake and aqueous solution combining any pharmaceutically acceptable carrier, excipient, stabilizer and the glycosylated human Interferon alpha isoform having a desired purity. A

preparation for parenteral administration can be prepared by combining the glycosylated human Interferon alpha isoform with a pharmaceutical carrier into a formulation, which can be administered (solution, suspension or emulsion).

The pharmaceutically acceptable carrier, excipient or stabilizer do not shown toxicity to a patient who receiving them at a dose and concentration to be administered and are compatible with other ingredients. For example, the preparation should not contain an oxidant or other substances, which are known as being harmful to a polypeptide.

Suitable carriers include buffers such as phosphoric acid, citric acid and other organic acids; antioxidants such as ascorbic acid; low-molecular polypeptides; proteins such as serum albumin, gelatin and immunoglobulin; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, arginine or lysine; monosaccharides such as mannose or dextrin, disaccharides, other carbohydrates; chelating factors such as EDTA; metal ions such as zinc, cobalt or copper; sugar alcohols such as manitol or sorbitol; salt-forming counter ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

In order to use the glycosylated human Interferon alpha isoform for therapeutic administration, it should be sterilized. The sterilization can be readily accomplished by filtration through a sterile filtration membrane.

The therapeutic composition of the glycosylated human Interferon alpha isoform is typically stored in a container having a sterile access port, such as, for example, vascular injection bag having a cap through which a subcutaneous injection needle can pass or vial. The human Interferon alpha will be stored as an aqueous solution or lyophilized preparation

in a single dose or multi-dose container, for example, a sealed vial or an ampoule. In case of the lyophilized preparation, 5 ml of sterilized and filtered 1%(w/v) human Interferon alpha aqueous solution is filled in a 10 ml-vial and the mixture is lyophilized. The injection can be prepared by reconstruction of the lyophilized human Interferon alpha with bacteriostatic Water-for-Injection.

The glycosylated human Interferon alpha isoform can be directly administered to animals by a proper technology including paranteral administration, or locally or systemically administered. A particular administration route can be determined depending on, for example, a patient's case history including side effects which are recognized or expected by the human Interferon alpha. Examples of the parenteral administration include subcutaneous, intramuscular, intravascular, intraarterial, intraperitoneal administration. Most preferably, the administration is carried out by sustained injection (ex. a mini pump such as osmosis pump) or injection through, for example, intravascular or subcutaneous route. The glycosylated human Interferon alpha isoform is preferably administered subcutaneously.

The glycosylated human Interferon alpha isoform is administered to a patient in a therapeutically effective amount. The term " therapeutically effective amount" can be defined as an amount sufficient to show a desired therapeutic effect in a given condition and administration method. The human Interferon alpha composition for treatment should be prepared and administered considering particular conditions to be treated, clinical conditions of individual patients (particularly side effects upon treatment with human Interferon alpha along), delivery location of the glycosylated human Interferon alpha

isoform, administration method, administration schedule, other factors known to those skilled in the art and being consistent with preferred medical practices. The therapeutically effective amount in the treatment with the glycosylated human Interferon alpha isoform is determined by the foregoing matters. A daily effective amount of the glycosylated human Interferon alpha isoform according to the present invention is in the range of about 2×10^6 units to 500×10^6 units.

Now, the present invention will be described in further detail by the following examples. However, the examples are only for illustration of the present invention and the present invention is not limited thereto.

<Example 1>

Preparation of human Interferon alpha gene

As human Interferon alpha gene, modified Interferon alpha-producing strain possessed by the applicant was used. The Interferon alpha gene possessed by the applicant did not comprise the whole sequence for expression in E. coli. Therefore, PCR using a chemically synthesized oligodeoxynucleotide was conducted to prepare the whole sequence. The human Interferon alpha gene without the whole sequence was amplified using PiaE5-1 and IFN-A5' synthetic oligodeoxynucleotide. The amplified DNA segment was amplified by PCR using a synthetic oligodeoxynucleotide of PiaE5-2 and IFN-A3' to introduce the full-length signal sequence at 5'-end of human Interferon alpha gene. The used synthetic oligodeoxynucleotide are shown in Table 1.

Table 1

Synthetic oligodeoxynucleotide used as primer for construction of whole sequence

Primer Name	primer sequence	SEQ ID NO
PiaE5-1	5-'GTGCTCAGCTGCAAGTCAAGCTGCTCTGTGGGCTGTGATCTGCCTCAAACCCAC-3'	1
PiaE5-2	5'-ATGGCCTTGACCTTTGCTTTACTGGTGGCCCTCCTGGTGCTCAGCTGCAAGTCA-3'	2
IFN-A5'	5'-TCCCAAGCTTATGGCCTTGACCTTTGCTTTACTG-3'	3
IFN-A3'	5'-TGGGATCCTCATTCCTTACTTCTTAAACTTTCTTG-3'	4
HisEK:1	5'-AAGCTTCCCATGGGGGGTTCTCATCATCATCATCATCATGGG -3'	5
HisEK:2	5'-CATCATCATCATCATCATGGGGACGATGACGATAAG -3'	6
Alpha: 1	5'-ACCCCCCATGGAGCCCACAGAGCAGCTTGA -3'	7
Alpha: 2	5'-GGGGACGATGACGATAAGTGTGATCTGCCTCAAACC -3'	8

<Example 2>

Selection of modification site on human Interferon alpha gene

In order to select a site for additional glycosylation on human Interferon alpha, the result of the reference [Walter(Structure(1996) vol.4, 1453)] was used. In selection of a site, firstly, the helical region in the amino acid sequence of human Interferon alpha protein was excluded (Fig. 1). From the sequence with the helical region excluded, a second site was selected, considering that No. 106 threonine residue of wild-type Interferon has O-type glycosylation in three-dimension. From the secondly selected site, a site where N-type glycosylation could be readily converted to a motive was finally selected.

As shown in Fig. 1, the sites to attempt modification for addition of an additional

glycosylation site was L26, H34 and F36, and K134, in which 26th leucine was modified with asparagine, 34th histidine and 36th phenylalanine were modified with asparagine and serine, and the 134th lysine was modified with asparagine. There are shown synthetic oligodeoxynucleotides used for this experiment. The direction of the arrow represents of 5'-> 3' direction of respective oligodeoxy nucleotides.

In order to purify human Interferon alpha protein, an additional amino acid sequence (HisEK) was inserted between the pre-sequence and the amino acid sequence of mature human Interferon alpha protein. The amino acid sequence was M-G-G-S-H-H-H-H-H-H-G-D-D-D-D-K-. By inserting this amino acid sequence, expressed human Interferon alpha derivative protein can isolated by metal affinity column chromatography. The isolated protein was treated with enterokinase and subjected to metal affinity column chromatography to obtain only human Interferon alpha derivative protein.

The insertion of HisEK sequence was conducted by amplifying DNA at the pre-sequence region by PCR with IFN-A5 and alpha:1 primer, followed by digestion with restriction enzyme NcoI. Then, the mature human Interferon alpha gene region was primarily amplified with alpha:2 and IFN-A3. The resulting DNA segment was secondarily amplified with HisEK:2 and IFN-A3 and then with HisEK:1 and IFN-A3 to obtain a DNA segment. The resulting DNA segment was digested with restriction enzyme NcoI and the resulting two DNA segments were joined using T4 DNA ligase.

The joined human Interferon alpha gene was again amplified by PCR using IFN-A5 and IFN-A3 primers. The amplified DNA segment was digested with restriction enzyme HindIII and BamHI and inserted to pcDNA3.1Hygro+ plasmid vector, which had

been digested with the same restriction enzymes, using T4 DNA ligase to form an expression vector.

<Example 3>

Construction of human Interferon alpha isoform

A gene encoding human Interferon alpha having at least one amino acid modified to provide an additional glycosylation site can be modified by PCR using a synthetic oligodeoxynucleotide as a primer. The used synthetic oligodeoxynucleotide are shown in Table 2.

Table 2

Synthetic oligodeoxynucleotide used for production of additional glycosylation

primer name	primer sequence	SEQ ID NO
L26N1	5'-GCACAGATGAGGCGCATCTCTAACTTCTCCTGCTTGAAGGACAGA-3'	9
L26N2	5'-TCTGTCCTTCAAGCAGGAGTTAAGAGAGATGCGCCTCATCTGTGC-3'	10
H34NF36S:1	5'-TTGAAGGACAGAAACGACAGCGGATTTCCCCAG-3'	11
H34NF36S:2	5'-CTTCATCAGGGGAGTCTCGTTCACCCCCACCCC-3'	12
K134N1	5'-ACTCTCTATCTGAAAGAGAAGAACTACAGCCCTTGTGCCTGGGAG-3'	13
K134N2	5'-CTCCCAGGCACAAGGGCTGTAGTTCTTCTCTTTCAGATAGAGAGT-3'	14
IFN-A5'	5'-TCCCAAGCTTATGGCCTTGACCTTTGCTTTACTG-3'	15
IFN-A3'	5-'TGGGATCCTCATTCCTTACTTCTTAAACTTTCTTG-3'	16

(1) Construction of L26N modified human Interferon alpha isoform (Fig. 3)

The human Interferon alpha gene obtained from Example 1 were amplified by PCR with synthetic oligodeoxynucleotide primers, IFN-A5' and L26N2, L26N1 and IFN-A3' to prepare DNA segments. Each of the prepared DNA segments was purified, denatured with 0.2M NaOH/2mM EDTA and subjected to PCR to prepare a gene with an amino acid at a desired site changed (Leu→Asn). As a result, two DNA segments substituted with a codon corresponding to asparagine instead of leucine at the No. 26 amino acid position were obtained. The two DNA segments were subjected to secondary PCR using a primer pair of IFN-A5' and IFN-A3' to obtain a modified gene of IFN-alpha-L26N, in which 26th amino acid is modified with asparagines so that an additional glycosylation can take place.

(2) Construction of H34NF36S modified human Interferon alpha derivative (Fig. 4)

Using the same method for the L26N modified modified human Interferon alpha derivative, human Interferon alpha gene was amplified by PCR with synthetic oligodeoxynucleotides IFN-A5 and H34NF36S:2, and H34NF36S:1 and IFN-A3 to prepare DNA segments.

Each of the DNA segments was purified and subjected to the same method as described above to prepare IFN-alpha H34NF36S modified gene, in which histidine at 34th amino acid position was changed to asparagine and phenylalanine at 36th amino acid position is changed to serine.

(3) Construction of K134N modified human Interferon alpha isoform (Fig. 5)

Using the same method for the L26N modified modified human Interferon alpha derivative, human Interferon alpha gene was amplified by PCR with synthetic oligodeoxynucleotides IFN-A5' and K134N2, and K134N1 and IFN-A3' to prepare DNA segments.

As a result, as shown in Fig. 4, two DNA segments substituted with a codon corresponding to asparagine instead of lysine at 134th amino acid position were obtained.

The two DNA segments were subjected to secondary PCR using a primer pair of IFN-A5' and IFN-A3' to obtain a modified gene of IFN-alpha-K134N, in which 134th amino acid is modified with asparagine so that an additional glycosylation can take place.

(4) Construction of human Interferon alpha derivative with both L26N and H34NF36S modified (Fig. 6)

The same method for the L26N modified modified human Interferon alpha derivative was followed using H34NF36S modified human Interferon alpha derivative.

(5) Construction of human Interferon alpha isoform with both L26N and K134N modified (Fig. 7)

The same method for the L26N modified modified human Interferon alpha derivative was followed using K134N modified human Interferon alpha isoform. In other words, 134th position was modified by the same method as shown in Fig. 5 and using the

product as a template, 26th position was modified by the same method as shown in Fig. 3. As a result, a human Interferon alpha gene with two sites modified at the same time was obtained.

<Example 4>

Transfection into CHO cell and expression

In a 60mm cell culture dish, CHO cells (DG44) were raised to 40-80% confluent (1-4 X 10^5cell/60mm dish). 3 $\mu\ell$ of Superfectin reagent (BM) and 97 $\mu\ell$ of cell culture medium (α -MEM with media, serum-free, antibiotic-free) were thoroughly mixed and human Interferon alpha derivative expression vector DNA (0.1 $\mu g/\mu\ell$ or more, about 2 μg) and vector pLTRdhfr26 (ATCC37295, about 0.2 μg) containing dhfr were added thereto. After the reaction was left for 5 to 10 minutes at room temperature and added to the prepared cells. After one day, the medium was exchanged with a memdium containing 200 $\mu g/m\ell$ of hygromycine (α -MEM without media, 10% FBS) and cultured for about 7 to 10 days. In the medium containing hygromycine at a concentration of 200 $\mu g/m\ell$, cell lines with human Interferon alpha derivative introduced were selected. Each of the selected cell lines was cultured and confirmed for expression of human Interferon alpha derivative by using a human Interferon alpha (Hu-IFN-α) Multi-Specific ELISA Kit (PBL, Product No. 41105-1;).

<Example 5>

Purification of human Interferon alpha derivative

The human Interferon alpha derivatives expressed in CHO cells were purified by condensing the culture fluid using Centriprep (Mw Cut 10,000, Milipore) and subjecting to metal affinity method using ProBond Purification System (Invitrogene).

<Example 6>

Pharmacokynetic test in rat

In order to confirm whether candidates can sustained in actual living bodies, Sprague Dawley rats were used. Amimals were injected with human Interferon derivative in a dose of 1 X 10^6 U/Kg body weight. Each group possessed 4 animals. In order to confirm blood concentration, blood was taken every 30 minutes. The blood samples were analyzed on Human Interferon Alpha(Hu-IFN-α) Multi-Specific ELISA Kit (PBL).

INDUSTRIAL APPLICABILITY

The glycosylated human interferon alpha isoform according to the present invention can has increased in vivo stability and thereby, reduce dose in clinical applications and the frequency of administration.

While the present invention has been described with reference to the particular illustrative embodiments, it is not to be restricted by the embodiments but only by the appended claims. It is to be appreciated that those skilled in the art can change or modify the embodiments without departing from the scope and spirit of the present invention.

What Is Claimed Is:

1. An amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

2. The an amino acid-modified human Interferon alpha isoform according to claim 1, which has 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or has all of these modifications.

3. A gene encoding an amino acid-modified human Interferon alpha isoform having at least one of the Asn-X-Ser/Thr(N-X-S/T) sequence formed at the following amino acid residue positions so that glycosylation takes place at these sites:

-Cys1-Ser8(Cys1-Asp-Leu-Pro-Gln-Thr-His-Ser8);

-Arg22-Thr52(Arg22-Arg-Ile-Ser-Leu-Phe-Ser-Cys-Leu-Lys-Asp-Arg-His-Asp-Phe-Gly-Phe-Pro-Gln-Glu-Glu-Phe-Gly-Asn-Gln-Phe-Gln-Lys-Ala-Glu-Thr52);

-Ser68;

-Asp77;

-Lys134-Ser137(Lys134-Tyr-Ser137); and

-Gln158-Glu165(Gln158-Glu-Ser-Leu-Arg-Ser-Lys-Glu165).

4. The gene according toclaim 3, wherein the human Interferon alpha has 26th leucine modified with asparagine, 34th histidine and 36th phenylalanine modified with asparagine and serine, respectively, or 134th lysine modified with asparagine, or has all of these modifications.

5. A method for producing a glycosylated human Interferon alpha isoform comprising the steps of: culturing a eukaryotic host cell transformed or transfected with an expression vector comprising a gene encoding the Interferon alpha isoform according to claim 3 or 4 and isolating an glycosylated human Interferon alpha isoform from the culture.

6. A pharmaceutical composition comprising the glycosylated human Interferon alpha isoform according to claim 1 or 2 and a pharmaceutically acceptable carrier.

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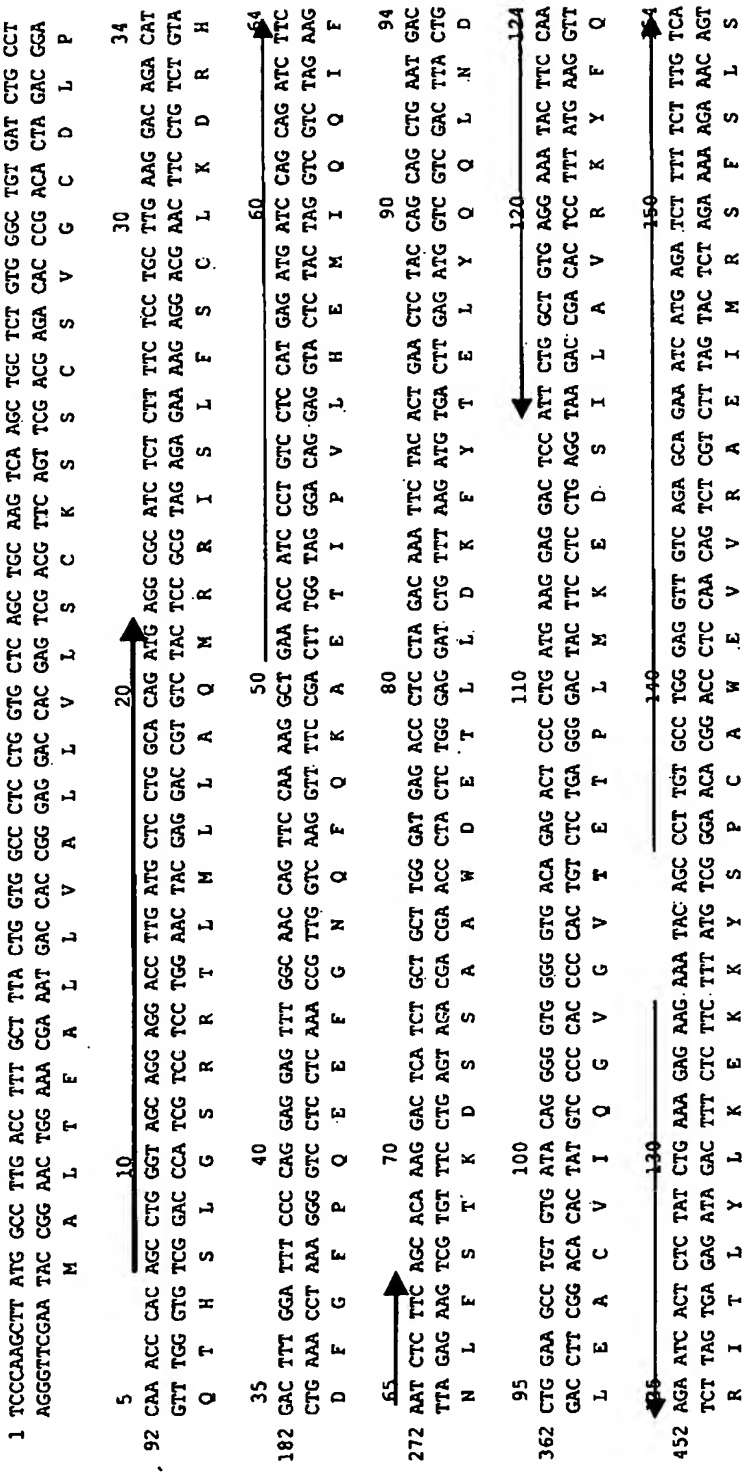
Fig. 1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M A L T F A L L L V A L L V L S C K S S C S S V G C D L P
92 CAA ACC CAC AGC CTG GGT AGC AGG AGG ACC TTG ATG CTC CTG GCA CAG ATG AGG CGC ATC TCT CTT TTC TCC TGC TTG AAG GAC AGA CAT
GTT TGG GTG TCG GAC CCA TCG TCC TCC TGG AAC TAC GAG GAC CGT GTC TAC TCC GCG TAG AGA GAA AAG AGG ACG AAC TTC CTG TCT GTA
Q T H S L G S R R T L M L L A Q M R R I S L F S C L K D R H
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CTG AAA CCT AAA GGG GTC CTC CTC AAA CCG TTG GTC AAG GTT TTC CGA CTT TGG TAG GGA CAG GAG GTA CTC TAC TAG GTC GTC TAG AAG
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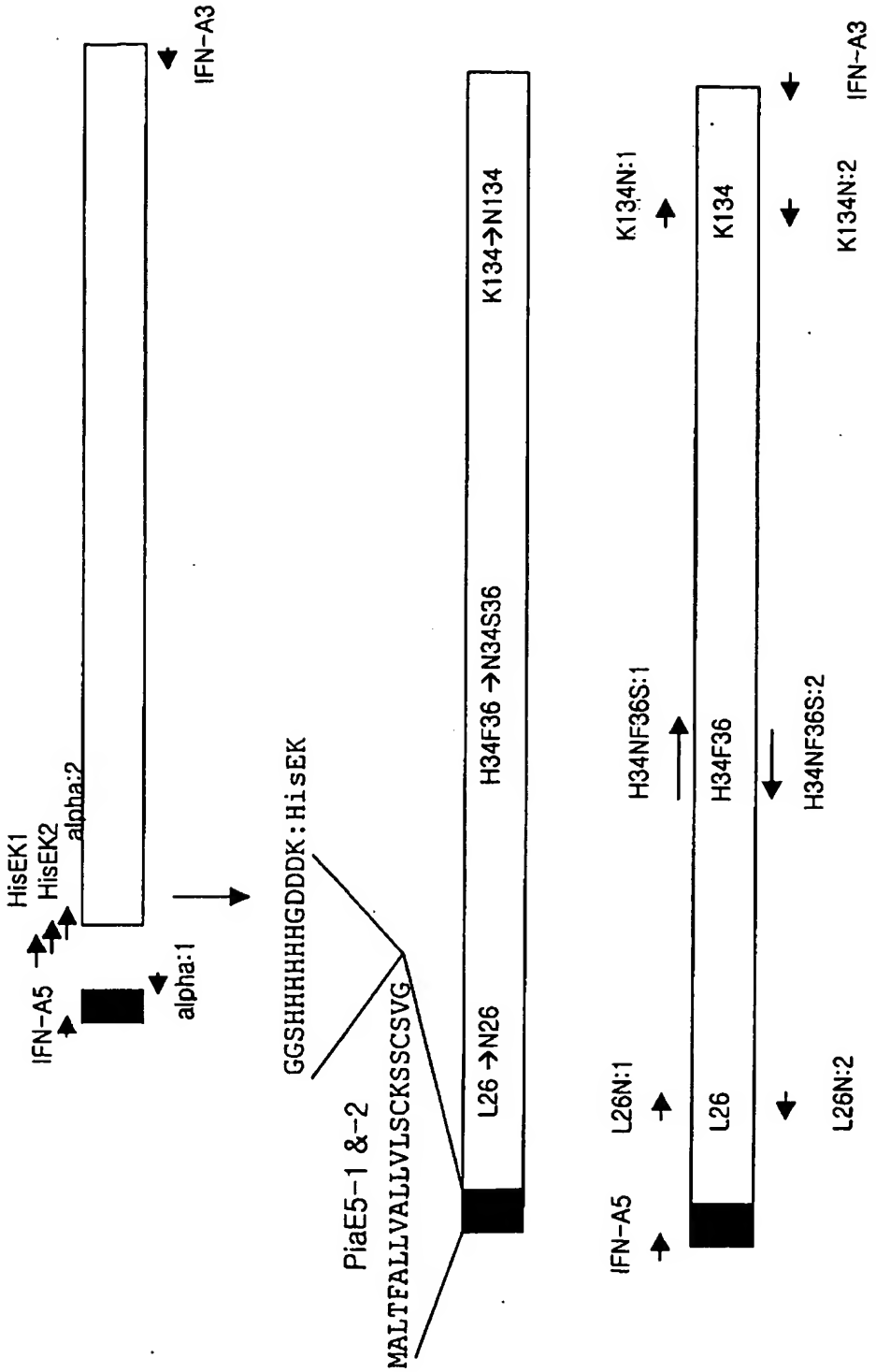
Fig. 2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L26 →N26
H34F36 →N34S36
K134→N134
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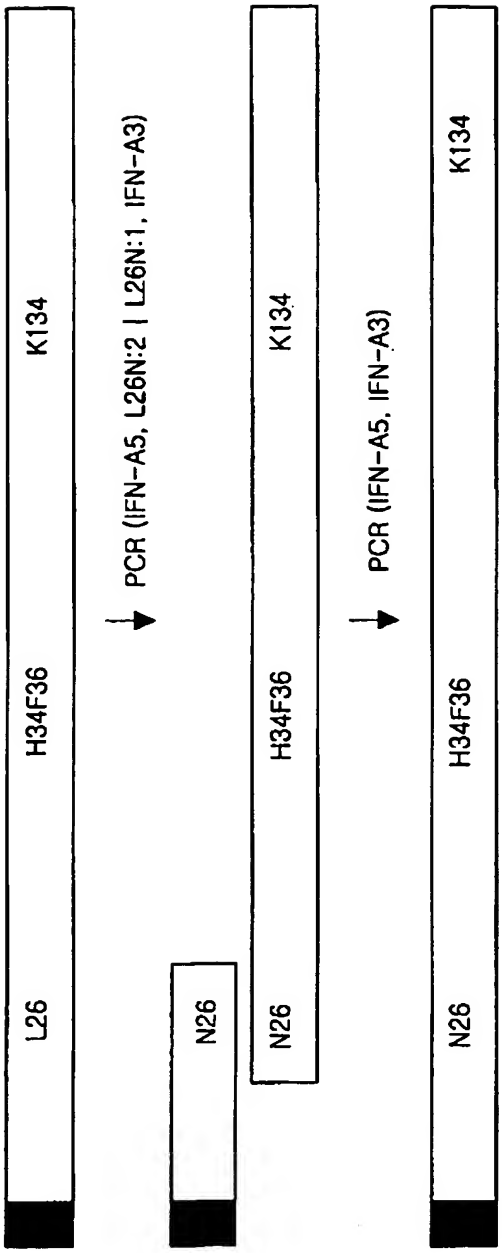
Fig. 3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K134

Fig. 4

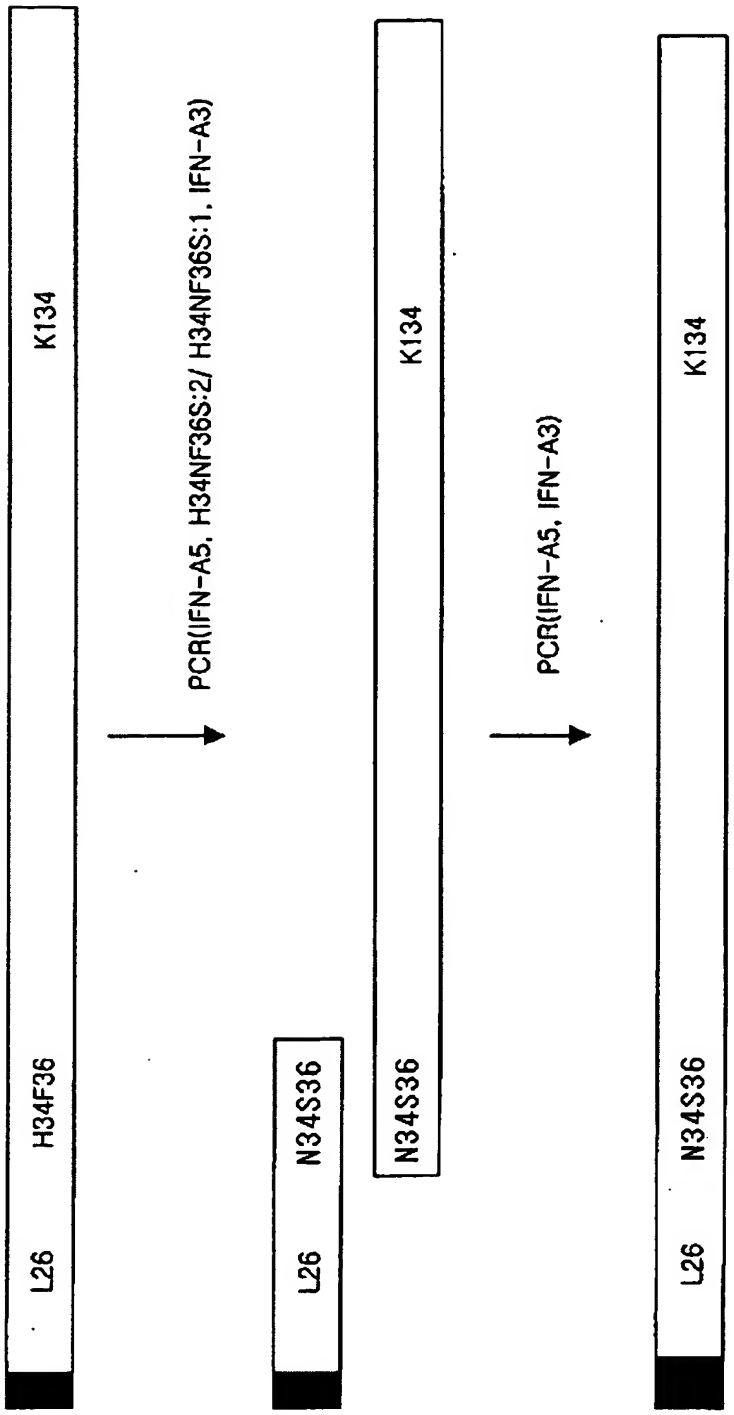
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Fig. 5

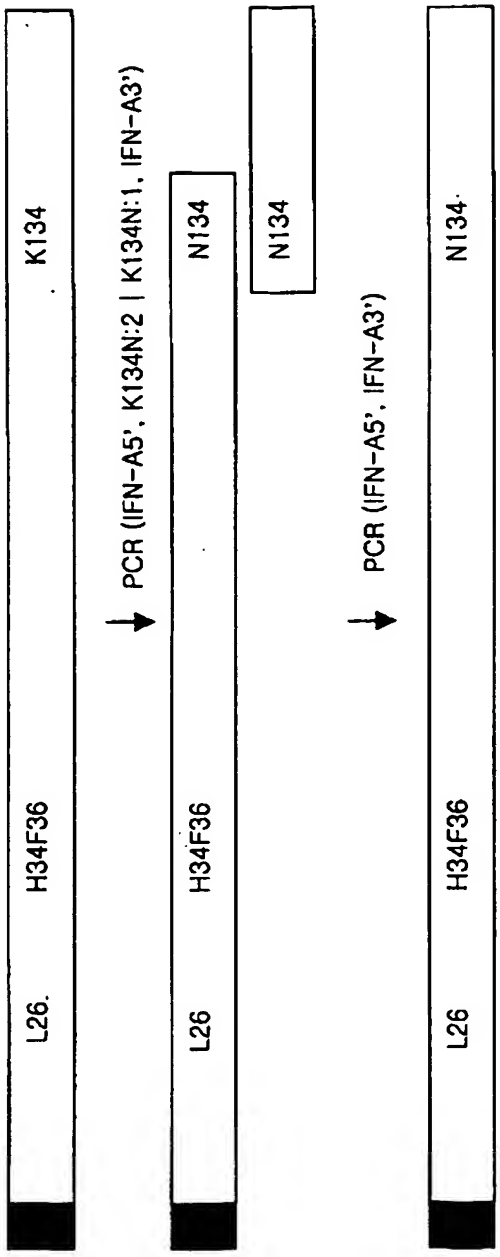
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Fig. 6

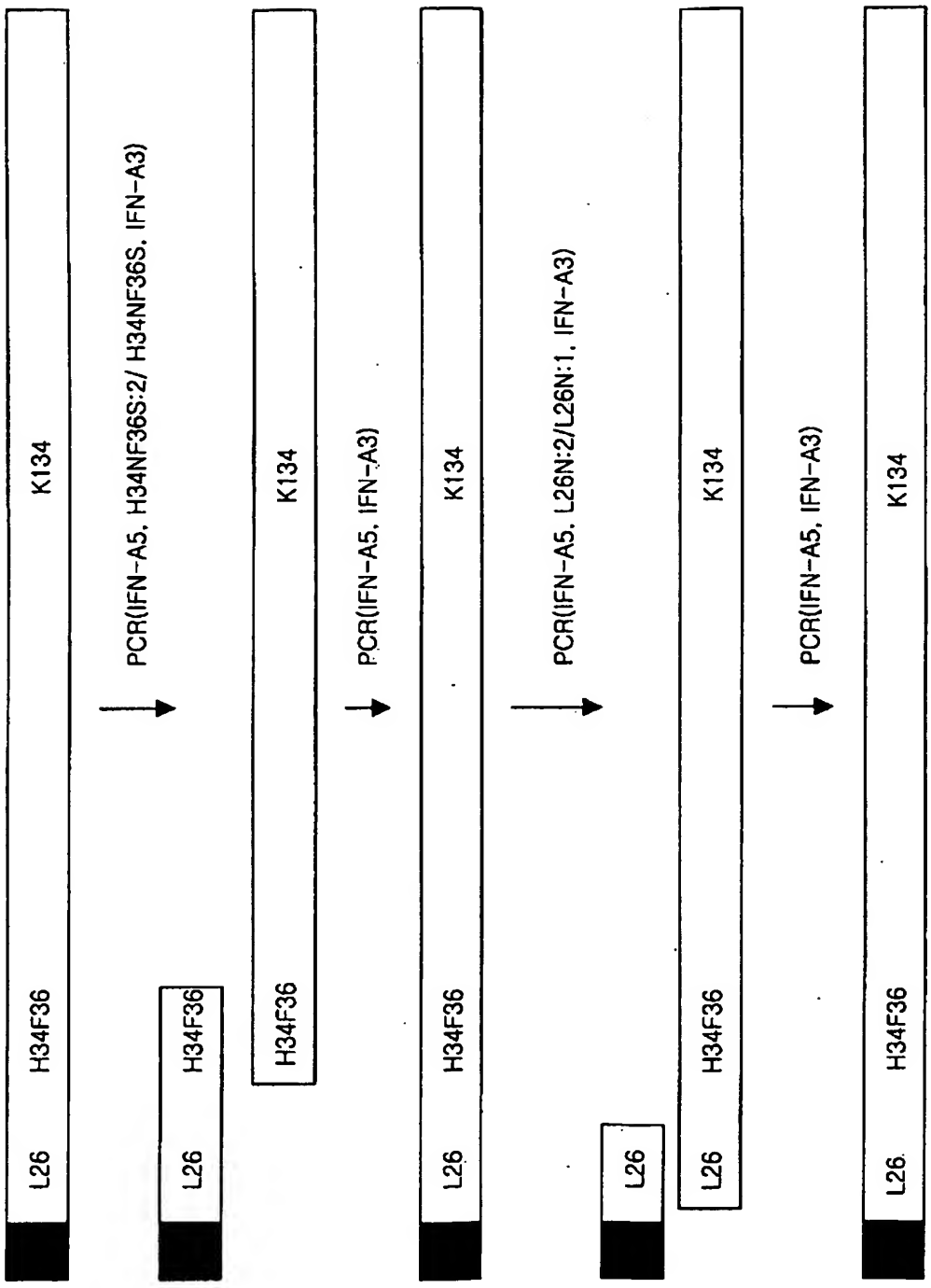
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Fig. 7

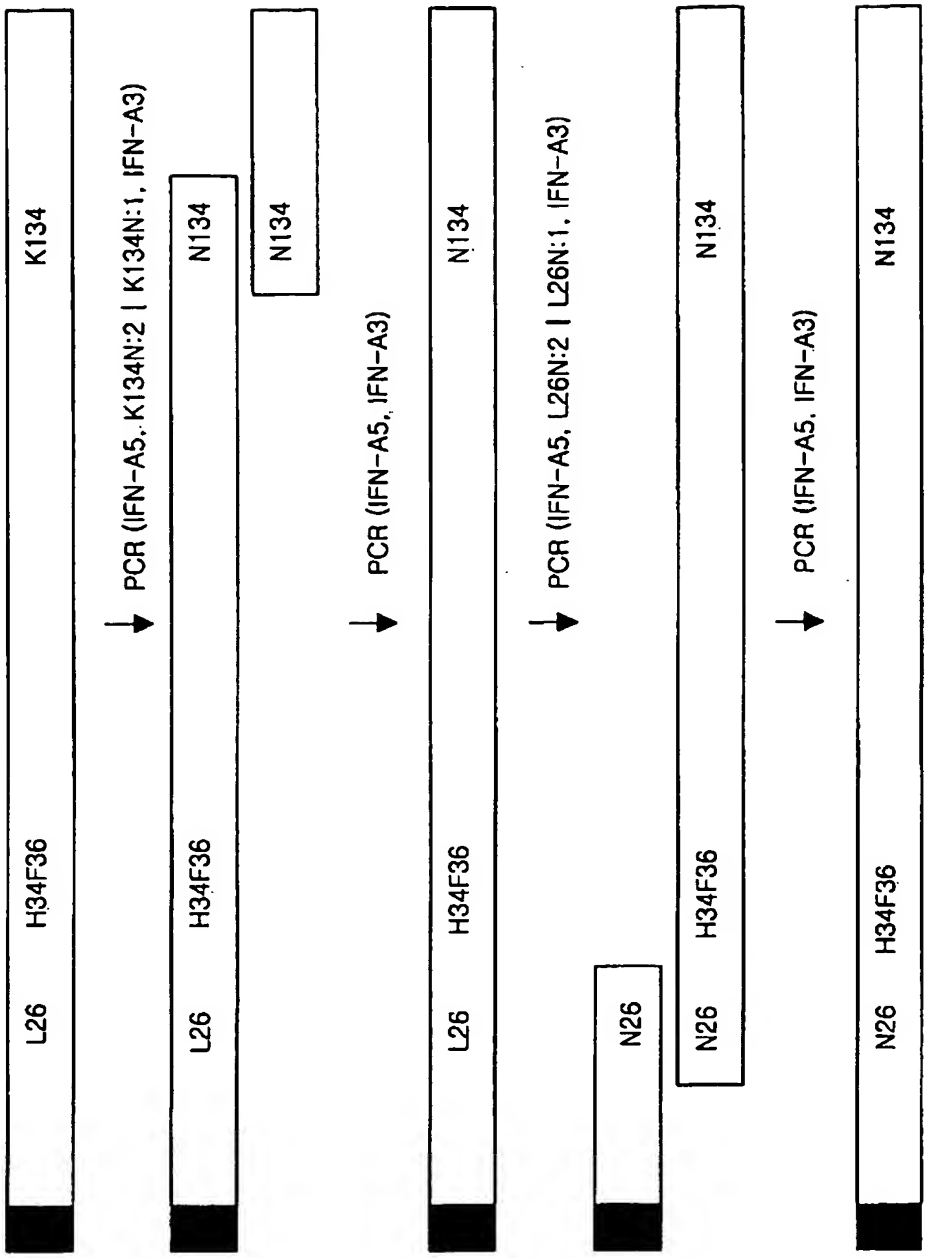
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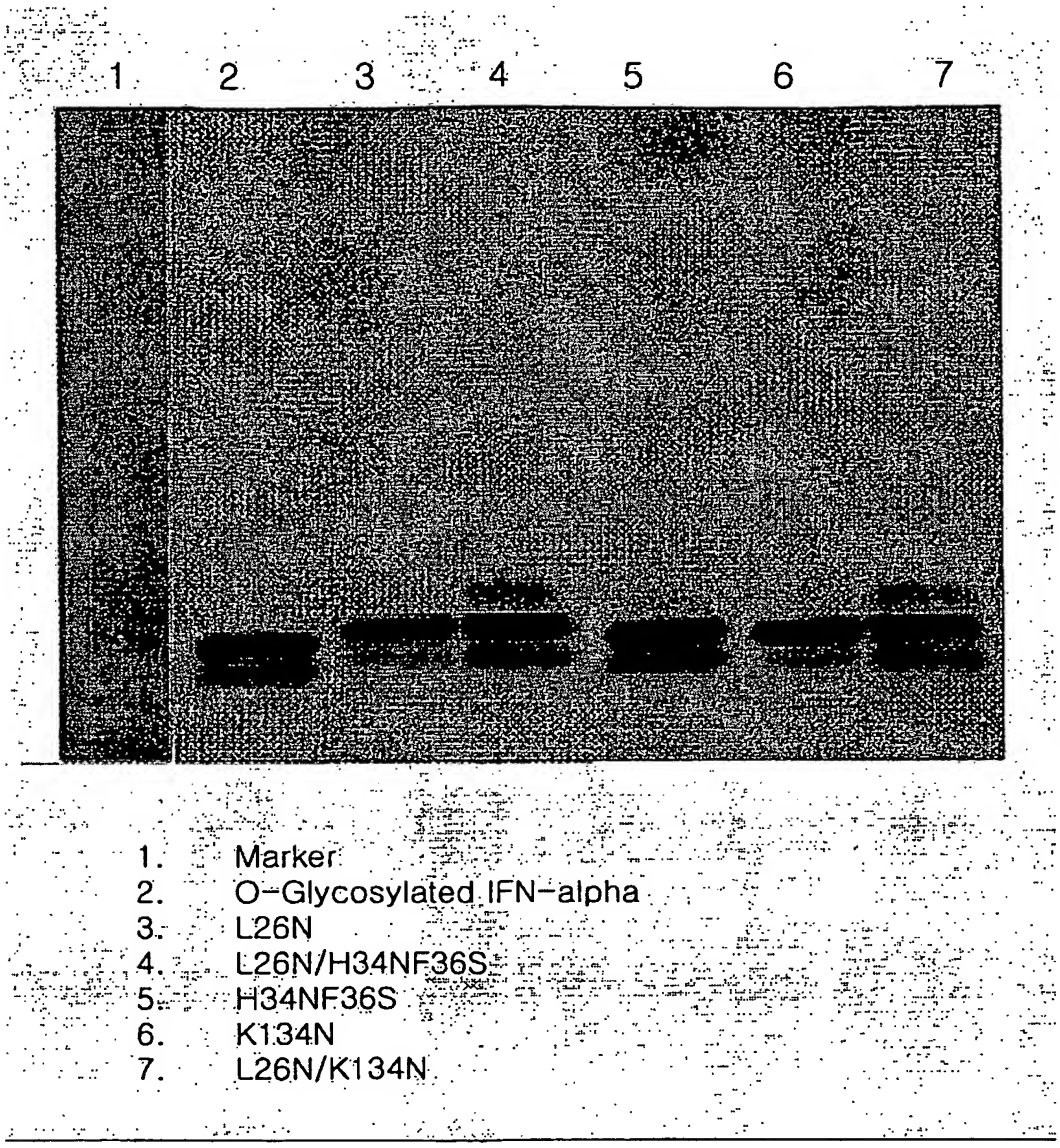
Fig. 8



1. Marker
2. O-Glycosylated IFN-alpha
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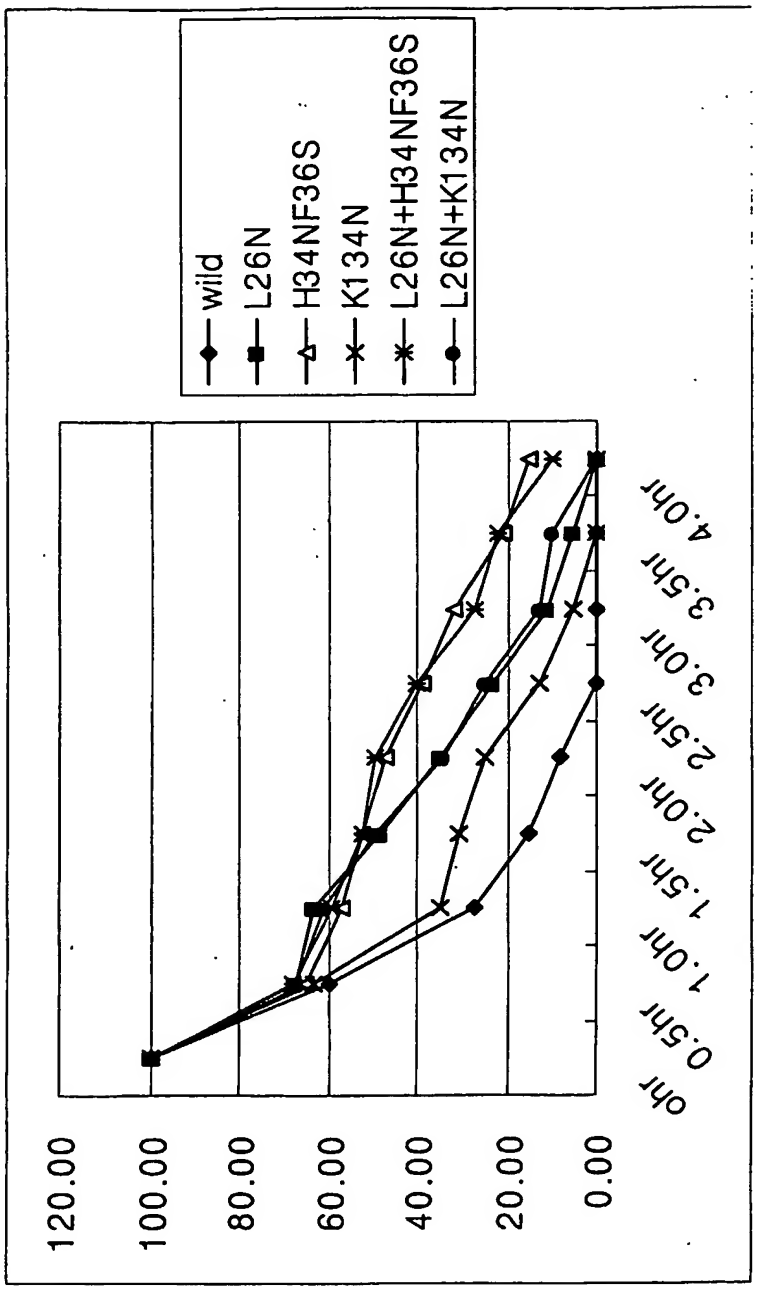
4. L26N/H34NF36S
5. H34NF36S
6. K134N
7. L26N/K134N

Fig. 9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Sequence Listing

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01765

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C07K 14/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korea Patent and Applications for Inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

PubMed, CA, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	James D.C., et al. "N-glycosylation of recombinant human interferon-gamma produced in different animal expression systems" Biotechnology (N. Y.)., 1995, Vol. 13(6): pages 592-596, see abstract.	1-6
Y	Sareneva T., et al. "Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma" Biochem. J., 1994, Vol. 303(Pt 3): pages 831-840, see abstract.	1-6
A	Nyberg G.B., et al. "Metabolic effects on recombinant interferon-gamma glycosylation in continuous culture of Chinese hamster ovary cells" Biotechnol. Bioeng., 1999, Vol. 62(3): pages 336-347, see abstract.	1-6
A	Nyman T.A., et al. "Identification of nine interferon-alpha subtypes produced by Sendai virus-induced human peripheral blood leucocytes" Biochem. J., 1998, Vol.329(Pt 2): pages 295-302, see abstract.	1-6

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 DECEMBER 2003 (19.12.2003)	19 DECEMBER 2003 (19.12.2003)

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
PARK, JEONG UNG
Telephone No. 82-42-481-8159

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